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# **Cyclosporine population pharmacokinetics in pediatric renal transplant recipients**

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**ACADEMIC DISSERTATION**

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## ABBREVIATIONS

3'UTR	3'-untranslated region
5'UTR	5'-untranslated region
<i>ABCB1</i>	Gene encoding the multidrug resistance protein (MDR) 1
<i>ABCC2</i>	Gene encoding the multidrug resistance-associated protein (MRP) 2
ANOVA	Analysis of variance
AUC	Area under the concentration-time curve
b.i.d.	Twice daily dosing
BSA	Body surface area
C <sub>0</sub>	Trough concentration
C <sub>2</sub>	Concentration two hours after the dose
CAN	Chronic allograft nephropathy
CL	Clearance
C <sub>max</sub>	Maximum concentration
CV	Coefficient of variation (=standard deviation/mean)
CYP	Cytochrome P450
CYP3A4	Cytochrome P450 3A4
CYP3A5	Cytochrome P450 3A5
EBE	Empirical Bayes estimate
F	Oral bioavailability
FO	First order estimation algorithm
FOCE	First order conditional estimation algorithm
FOCE INTER	First order conditional estimation with interaction algorithm
FPIA	Fluorescence polarization immunoassay
HPLC	High pressure liquid chromatography
i.v.	Intravenous
IIV	Inter-individual variability
IOV	Inter-occasion variability (within-patient variability)
MDR1	Multidrug resistance protein 1 (P-glycoprotein)
MRP	Multidrug resistance-associated protein
<i>NR1I2</i>	Gene that encodes the nuclear receptor PXR

## ABBREVIATIONS

OATP	Organic anion transporting polypeptide
OFV	Objective function value
p.o.	Oral
PXR	Pregnane X receptor
$Q_H$	Hepatic blood flow
SD	Standard deviation
<i>SLCO1B1</i>	Gene that encodes the OATP1B1 protein
SNP	Single nucleotide polymorphism
RIA	Radioimmunoassay
t.i.d.	Thrice daily dosing
$t_{1/2}$	Elimination half-life
TDM	Therapeutic drug monitoring
$T_{max}$	Time to maximum concentration
TVCL	Typical value of clearance
TX	Transplantation
Vd	Volume of distribution
$\varepsilon$	Difference between individual prediction and observation (residual error)
$\eta_i$	Difference between the population parameter and the individual parameter estimate
$\theta$	Fixed-effect parameter (typical value)



## LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the three original contributions listed below. They will be referred to by Roman numerals I, II, and III in the text.

- I Fanta S, Jönsson S, Backman JT, Karlsson MO, Hoppu K. Developmental pharmacokinetics of ciclosporin – a population pharmacokinetic study in paediatric renal transplant candidates. *Br J Clin Pharmacol* 2007;64:772-84.  
Erratum in: *Br J Clin Pharmacol* 2008;65:973.
- II Fanta S, Niemi M, Jönsson S, Karlsson MO, Holmberg C, Neuvonen PJ, Hoppu K, Backman JT. Pharmacogenetics of cyclosporine in children suggests an age-dependent influence of *ABCB1* polymorphisms. *Pharmacogenet Genomics* 2008;18:77-90.
- III Fanta S, Jönsson S, Karlsson MO, Niemi M, Holmberg C, Hoppu K, Backman JT. Long-term changes in cyclosporine pharmacokinetics after renal transplantation in children: evidence for saturable presystemic metabolism and effect of *NR1I2* polymorphism. *J Clin Pharmacol*. In Press.

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## ABSTRACT

Cyclosporine is an immunosuppressant drug that has a narrow therapeutic index and also large variability in its pharmacokinetics. It is likely that the inter- and intra-individual variability in cyclosporine pharmacokinetics and dose requirements is even higher in children than in adults as a result of variations in biological maturation status. In order to improve cyclosporine dose individualization in children, we used population pharmacokinetic modeling to study the effects of developmental, clinical, and genetic factors on cyclosporine pharmacokinetics in a total of 176 subjects (age range: 0.36–20.2 years) before and up to 16 years after renal transplantation. Pre-transplantation test doses of cyclosporine were given intravenously (3 mg/kg) and orally (10 mg/kg), on separate occasions, then followed by blood sampling for 24 hours (n=175). Cyclosporine concentration was quantified after transplantation in a total of 137 patients at: trough, two hours post-dose, or with dose-interval curves. Of these studied patients 104 were genotyped for 17 putatively functionally significant sequence variations in the *ABCB1*, *SLCO1B1*, *ABCC2*, *CYP3A4*, *CYP3A5*, and *NR1I2* genes. Pharmacokinetic modeling was performed using the nonlinear mixed effects modeling computer program, NONMEM.

A 3-compartment population pharmacokinetic model that had first order absorption without lag-time was used to describe the data. The most important covariate that affected systemic clearance and distribution volume was allometrically scaled body weight, i.e. body weight<sup>3/4</sup> for clearance and absolute body weight for volume of distribution. The clearance adjusted for absolute body weight declined with age. Pre-pubertal children (<8 years) had approximately 25% higher clearance/body weight values (L/h/kg) than did older children. Adjustment of clearance for allometric body weight removed this relationship to age after the first year of life. This finding is consistent with a gradual reduction in relative liver size towards adult values, and a relatively constant CYP3A content in the liver from about 6–12 months of age to adulthood.

The other significant covariates that affected cyclosporine clearance and volume of distribution were hematocrit, plasma cholesterol, and serum creatinine, which combined explained up to 20%–30% of inter-individual differences before transplantation. After transplantation, their predictive roles diminished, as the variations in hematocrit, plasma cholesterol, and serum

creatinine also decreased. Before transplantation, no clinical or demographic covariates were found to affect oral bioavailability, and no systematic age-related changes in oral bioavailability were observed. After transplantation, older children who received cyclosporine twice daily as the gelatine capsule microemulsion formulation manifested about 1.25–1.3 times higher bioavailability for cyclosporine than did the younger children who received it in the liquid microemulsion formulation thrice daily. Moreover, the oral bioavailability of cyclosporine increased over 1.5-fold in the first month after transplantation, and thereafter gradually returned to its initial value within 1–1.5 years of transplantation. The largest cyclosporine doses were administered in the first 3–6 months after transplantation, and thereafter the single doses of cyclosporine were often smaller than 3 mg/kg. Thus, the results suggest that cyclosporine displays dose-dependent, saturable pre-systemic metabolism even at low single doses, whereas complete saturation of CYP3A4 and MDR1 (P-glycoprotein) renders cyclosporine pharmacokinetics dose-linear at higher doses.

The pre-transplantation oral bioavailability of cyclosporine poorly predicted the post-transplantation oral bioavailability value, which suggests a limited effectiveness of oral pre-transplantation studies whose objective is to estimate the oral starting dose of cyclosporine. Moreover, the within-patient variability of oral bioavailability was high (CV≈20%) throughout the post-transplantation time period. This suggests that frequent monitoring is necessary, particularly during the first months after transplantation.

No significant associations were found between genetic polymorphisms and cyclosporine pharmacokinetics before transplantation in that whole population for which genetic data were available (n=104). However, bioavailability of cyclosporine in children older than eight years (n=22), who were heterozygous and homozygous carriers of the *ABCB1* c.2677T or c.1236T alleles were respectively about 1.3 times or 1.6 times higher, than that for non-carriers. After transplantation, none of the *ABCB1* SNPs or any other SNPs were found to be associated with cyclosporine clearance or oral bioavailability in the study population, for those patients older than eight years, or in those younger than eight years. However, in those patients who carried the *NR1I2* g.-25385C–g.-24381A–g.-205\_–200GAGAAG–g.7635G–g.8055C haplotype, the bioavailability of cyclosporine was about one tenth lower, per allele, than in non-carriers. This effect was also significant in a subgroup of patients older than eight years. Furthermore, in those patients who

## ABSTRACT

carried the *NR1I2* g.-25385C–g.-24381A–g.-205\_-200GAGAAG–g.7635G–g.8055T haplotype, the bioavailability was almost one fifth higher, per allele, than in non-carriers.

These conclusions were made using a robust modeling approach with a large dataset that combined rich and sparse cyclosporine pharmacokinetic data respectively obtained before and after renal transplantation. Adult CYP3A activity seems to have been reached by the age of 6–12 months, and allometrically scaled body weight was found to be a good predictor of the hepatic clearance of cyclosporine. It may be possible to improve the individualization of cyclosporine dosing in children by accounting for the effects of developmental factors (body weight, liver size), time after transplantation, and cyclosporine dosing frequency/formulation. Further studies are required on the predictive value of genotyping for individualization of cyclosporine dosing in children.

## INTRODUCTION

At present renal transplantation is considered the standard care for children with end-stage renal disease. Currently, more than 200 children have received renal transplants in Finland. The first pediatric kidney transplantations in adolescent patients in Finland were carried out in the 1960s. However, in that time period, younger children with an end stage renal disease were not applicable for treatment (Huhtamies and Relander, 1997). The factors that enabled the beginning of kidney transplantation in younger children were the development of pre- and post-transplantation treatments. For example, the advances in dialysis treatment, especially in peritoneal dialysis (Rönnholm and Holmberg, 2006), and the discovery of cyclosporine in the 1970s (Borel, 1976; Petcher et al., 1976).

The use of cyclosporine in post-transplantation treatment dramatically increased renal allograft survival (by about 60% at one year post-transplantation) (Lancet, 1983; N Engl J Med, 1983). An important factor pertaining to the improved allograft survival in young renal transplant recipients was that cyclosporine treatment enabled adequate immunosuppression without the serious growth impairment previously associated with the use of large doses of glucocorticoids (Cooney et al., 1997). Unfortunately, cyclosporine is a drug with a narrow therapeutic index and has large variability in its pharmacokinetics (Kahan, 1989a). In order to protect patients from the adverse effects related to excessive concentrations: mainly nephrotoxicity, and also to ensure adequate immunosuppression to avoid acute rejection, therapeutic drug monitoring is used to monitor cyclosporine concentrations (Lindholm and Kahan, 1993). In addition to using therapeutic drug monitoring, an understanding of the individual, clinical and genetic factors that affect the variability in cyclosporine pharmacokinetics could help clinicians to anticipate better the need for dosing modifications of cyclosporine.

In Finland the incidence of NPHS1 (congenital nephrosis of the Finnish type) is high, at 1 in 8000 live births (Jalanko, 2007). The disease, which is caused by mutations in the NPHS1 gene (Patrakka et al., 2000), leads to heavy proteinuria and subsequent death without treatment. Early nephrectomy and supportive care followed by renal transplantation is currently the best

## INTRODUCTION

treatment for children born with NPHS1. This is why about 50% of the children who receive their first kidney graft in Finland are under the age of five years (Laine et al., 1998).

In young children studies pertaining to cyclosporine pharmacokinetics and the developmental aspects of cyclosporine pharmacokinetics have been conducted using only small numbers of subjects (Cooney et al., 1997; del Mar Fernandez De Gatta et al., 2002). Similarly the pharmacokinetics of other cytochrome P450 3A substrates are poorly characterized in young children (Björkman, 2006). Advances in pediatric pharmacologic research have underlined that the greatest changes in drug pharmacokinetics occur most rapidly in the first years of life (Johnson, 2003; Bartelink et al., 2006; Kennedy, 2008). Furthermore, genotype-phenotype associations may only be apparent when a gene of interest is fully expressed due to developmental factors. Thus, a DNA sequence variation that causes dysfunction in a drug transporter protein in adults may not have the same effect in children, if the transport protein in question is not being expressed at significant levels at the time (Stephenson, 2005).

Because renal transplanted children have to take immunosuppressive medication for the rest of their lives, the possible negative impact on renal function (Tantravahi et al., 2007; Srinivas and Meier-Kriesche, 2008), growth, and the risk of developing malignancies (Buell et al., 2006; McDonald et al., 2008) should be borne in mind and immunosuppression should be minimized (Tönshoff and Höcker, 2006; Matas, 2007; Srinivas and Meier-Kriesche, 2008). Therefore it is important to profoundly understand the individual factors that affect cyclosporine pharmacokinetics and to use this information to optimize the dosing for each individual.

The children treated at the pediatric organ transplantation unit of Helsinki University Central Hospital have undergone cyclosporine concentration monitoring since pediatric renal transplantation began in Finland in the late 1980s. The therapeutic drug monitoring data have been collected as a by-product of the clinical tapering of cyclosporine dosing as the main aim of the monitoring was to aid the treatment. However, the large amount of retrospective data collected can now offer an excellent opportunity to study the factors that affect the pharmacokinetics of cyclosporine in children. In order to extract the largest possible amount of knowledge from those data, a population pharmacokinetic modeling approach was used in the data analyses. Population pharmacokinetic modeling has marked advantages over conventional

analyses in studying data that is part sparse and part rich: as was the case with the collected therapeutic drug monitoring data (Davidian and Giltinan, 1995). In addition, the model based approach has the advantage of enabling clinically relevant dosing predictions with the final model.

In summary, this study was carried out to gain more understanding on the developmental, clinical, and genetic factors that affect cyclosporine pharmacokinetics in Finnish renal transplant recipients and to find ways to optimize further cyclosporine dosing.

## REVIEW OF LITERATURE

### 1. DRUG METABOLISM AND TRANSPORT

#### **Drug metabolism**

Drug metabolism occurs mainly in the liver, and the intestinal wall (Gibson and Skett, 2001). It also occurs to a lesser extent in the kidneys, lungs and skin (Krishna and Klotz, 1994). Drug metabolism is often grouped into phase I functionalization and phase II conjugation reactions. Phase I reactions include oxidation, reduction, and hydrolysis reactions, whereby a functional group is inserted into the parent compound, or the compound is broken down. Phase II reactions involve the addition of an endogenous compound, such as glucuronic acid, glutathione, or sulphate to the parent compound or phase I metabolite. For those drugs that undergo phase I and phase II biotransformation sequentially, the first phase generally plays a larger role in determining the rate of the elimination process. Drug metabolism often reduces the biological activity of the parent compound and makes lipophilic substances more hydrophilic and therefore easier to excrete from the body in the feces (bile) and in urine (Gibson and Skett, 2001).

#### **Cytochrome P450 enzymes**

The most important phase I metabolizing enzyme system is the Cytochrome P450 (CYP) enzyme family that catalyzes the metabolism of a large number of lipophilic endogenous and exogenous compounds (Wrighton and Stevens, 1992). Individual CYP enzymes are divided into families, subfamilies and specific iso-enzymes that are classified by their amino acid similarities. Although individual cytochrome P450 enzymes each have unique substrate specificity, considerable overlap also occurs (Wilkinson, 2005). Of the 57 identified human CYP enzymes, most CYP enzyme families have mainly endogenous roles. The most important CYP subfamilies in the metabolism of exogenous compounds are CYP1, CYP2 and CYP3. The CYP3A subfamily enzymes are abundant in the human liver and the small intestine and are involved in the metabolism of around 50% of all drugs (Wilkinson, 2005).



## CYP3A

The substrate specificity of CYP3A enzymes is wide and includes a broad variety of structurally diverse compounds ranging from small molecules, such as triazolam, to large molecules such as cyclosporine which has a molecular weight of about 3.5 times higher than that of triazolam (Kenworthy et al., 1999). The CYP3A subfamily consists of at least four iso-enzymes: CYP3A4, CYP3A5, CYP3A7 and CYP3A43 (de Wildt et al., 1999; Daly, 2006). CYP3A is the most abundantly expressed CYP subfamily in the liver and it accounts for about 30% of the total CYP content in the liver (Rowland Yeo et al., 2003). CYP3A proteins are also substantially expressed in the intestine, especially in the duodenum and the proximal jejunum (Kolars et al., 1994). Although the total CYP3A content in the intestine is smaller compared to that found in the liver, the tips of the duodenal and proximal jejunal villi are lined with mature CYP3A4-containing enterocytes, which are readily exposed to any drug molecules dissolved in the gastric and intestinal contents. This localization of CYP3A4 and its high content in the enterocytes (von Richter et al., 2004) support the concept that drug metabolism in the intestinal wall substantially contributes to the overall first-pass metabolism of many CYP3A4 substrates (Kivistö et al., 2004), such as midazolam (Paine et al., 1996) and cyclosporine (Kolars et al., 1991). Little correlation exists between hepatic and intestinal CYP3A4 activities within individuals, suggesting independent regulation at the two sites.

The CYP3A5 iso-enzyme is 83% homologous with CYP3A4 and is also found in hepatic tissue and the small intestine, although usually at lower levels than CYP3A4 (Schuetz et al., 1989; Wrighton et al., 1989; Paine et al., 1997). CYP3A7 is the major CYP isoform that is detected in embryonic, fetal, and neonate liver. In contrast, CYP3A43 has minimal if any xenobiotic metabolizing activity and where detectable is expressed at low levels (Daly, 2006). The substrate specificities of CYP3A5 and CYP3A7 appear to be similar to that of CYP3A4, though with some differences (de Wildt et al., 1999). For instance, cisapride is metabolized by CYP3A4 but not by either CYP3A5 or CYP3A7 (Pearce et al., 2001).

## Drug transporters

Drugs can pass through the plasma membranes of cells passively by diffusion or by facilitated diffusion involving transport proteins. Active drug transport is mediated by transporter proteins as primary or secondary active transport (Giacomini and Sugiyama, 2006). Together the interplay of drug metabolizing enzymes and drug transporting proteins determines the absorption,

distribution, metabolism and excretion of a drug (Ho and Kim, 2005). In addition to transporting exogenous molecules, drug transporter proteins also have normal physiologic roles, in terms of transporting endogenous substances including sugars, lipids, amino acids, bile acids, steroids, and hormones (Ho and Kim, 2005). Drug transporting proteins are divided into influx and efflux transporters, based on the movement of the substrate: i.e. into or out of the cell.

### **OATP1B1**

Influx drug transporters belong to the super-family of solute carriers (SLCs) (Giacomini and Sugiyama, 2006) and these include the sodium-independent uptake transporters, organic anion transporting polypeptides (OATP). The OATP1B1 polypeptide, which is encoded by the *SLCO1B1* gene, is expressed mainly on the sinusoidal membrane of hepatocytes and its substrates include structurally diverse compounds such as: statins, benzylpenicillin, rifampin, enalapril, valsartan and methotrexate (Niemi, 2007). Cyclosporine is a relatively potent competitive inhibitor of OATP1B1, but has not directly been shown to be its substrate (Shitara et al., 2003; Kajosaari et al., 2005).

### **MDR1**

Efflux transporters belong to the ATP-binding cassette (ABC) transporter super-family, and include MDR1/ABCB1, and MRP2/ABCC2, which have been shown to be involved in drug disposition (Giacomini and Sugiyama, 2006). The MDR1 transporter (multidrug resistance protein 1, also known as the P-glycoprotein), is encoded by the *ABCB1* gene, and functions as an efflux pump by transporting its substrates from inside to the outside of the cell (Fromm, 2004). Moreover, MDR1 is situated in: the luminal membrane of enterocytes, the canalicular membrane of hepatocytes, the luminal membrane of the kidney proximal tubule cells, lymphocytes, the blood-tissue-barriers of the brain (blood-brain-barrier), the testis, and the placenta (Fromm, 2004). The MDR1 transports a broad variety of structurally diverse compounds. Most, but not all, MDR1 substrates are also substrates of CYP3A4 (Fromm, 2004). Due to the localization of MDR1 (on the apical surface) and CYP3A4 (intra-cellular) in the intestine, the function of MDR1 may allow CYP3A4 to have repeated and prolonged access to its substrate molecules, thus limiting the oral bioavailability of MDR1 and CYP3A4 substrates (Kivistö et al., 2004).

**MRP2**

MRP2 (multidrug resistance-associated protein 2), is encoded by the *ABCC2* gene. It is expressed on the apical membrane of hepatocytes, enterocytes, and renal proximal tubular cells. The MRP2 substrates include pravastatin, methotrexate, cisplatin, vinca alkaloids, hiv-protease inhibitors (Giacomini and Sugiyama, 2006). Cyclosporine is a relatively potent competitive inhibitor of MRP2, but has not been directly shown to be its substrate (Chen et al., 1999).

**Regulation of drug metabolism and transport**

The activities of drug transporters and CYP enzymes are governed by the induction, inhibition, and their constitutive expressions. Induction is a process by which prolonged exposure to an inducer compound causes an up-regulation in the amount of transporter or metabolizing enzymes. The induction process is usually mediated by the binding of the inducer compound to a nuclear receptor, which causes the increased transcription of the target gene and finally an increase in the rate of protein synthesis. On the other hand, inhibition is caused directly by an inhibitor compound that interacts with a drug transporter or metabolizing enzyme with the immediate result of reduced transporter or metabolizing enzyme activity (Lin and Lu, 1998; Ho and Kim, 2005).

The mechanism by which CYP3A4 is up-regulated involves intracellular binding of the inducer (drug compound, dietary agent, or hormone) to the nuclear receptor. This receptor, NR1I2, is also called the pregnane X receptor (PXR) or the steroid X receptor. Subsequently, it forms a heterodimer with the retinoid X receptor (RXR). The heterodimer then functions as a transcription factor by interacting with similar response elements located in the 5' regulatory region of the CYP3A4 gene. The end result is the increased synthesis of new CYP3A4 protein. The Pregnane X receptor has broad substrate specificity and thus may be activated by a large number of chemically diverse compounds found in the diet in addition to the therapeutic agents (Pelkonen et al., 1998; Wilkinson, 2005; Urquhart et al., 2007). Other nuclear factors that affect CYP3A4 expression include the constitutive androstane receptor (CAR), the glucocorticoid receptor (GR), the hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ), the farnesoid X receptor (FXR), and the vitamin D receptor (VDR) (Urquhart et al., 2007). CYP3A5 seems to be regulated similar to that described for CYP3A4, but the regulation of CYP3A7 and other 3A isoforms is less well characterized (Urquhart et al., 2007).

Like CYP3A4, MDR1 is also regulated by PXR. For example, rifampin co-administration significantly decreases digoxin levels via an inducing effect on MDR1 expression. Expression of MDR1 is higher in cells that stably express CAR than in cells that do not, thus suggesting the functional relevance of CAR-dependent activation of MDR1 (Burk et al., 2005; Gong et al., 2006). Multidrug resistance-associated protein 2 has been shown to be regulated by the nuclear receptors PXR, CAR, and FXR. Increased MRP2 expression has been noted after treatment with the following ligands for: PXR (rifampin, hyperforin), FXR (chenodeoxycholic acid), and by the CAR activator phenobarbital (Kast et al., 2002). Binding sites for PXR have been identified in the *SLCO1B1* promoters, but details of OATP1B1 induction are currently unclear (Teng and Piquette-Miller, 2008).

## 2. GENETIC FACTORS THAT CAUSE VARIABILITY IN DRUG METABOLISM AND TRANSPORT

### Pharmacogenetics

Pharmacogenetics is "*the study of variations in DNA sequence as related to drug response*", where drug response includes pharmacokinetics and pharmacodynamics (EMA, 2007). The expression and activity of drug metabolizing enzymes and drug transporters can increase or decrease, which leads to inter-individual variability in drug exposure and effect. These changes in expression and activity can be caused by DNA sequence variations (polymorphisms) in the genes that encode the drug metabolizing enzymes and drug transporters (Ho and Kim, 2005; Gardiner and Begg, 2006; Nebert et al., 2008).

### CYP3A inter-individual variability

Considerable variability exists in the expression of CYP3A in human small intestine and liver, and this is likely to contribute to the variable pharmacokinetics of intravenously, and especially orally administered drug substrates. The inter-individual variability in CYP3A4 drug-metabolizing activity has been estimated to be between 5- and 20-fold (Flockhart and Rae, 2003). Despite this, CYP3A activity is readily modulated by several factors, including drug administration. The activity of CYP3A can vary markedly among members of a given population, but its distribution seems to be continuous and unimodal. This suggests that multiple genes are involved in its regulation and individual genetic factors play a minor role. In fact, no common polymorphisms explaining the

variability in CYP3A4 activity have been identified. However, the rare (in Caucasians) *CYP3A4* g.-392A>G variation (*CYP3A4\*1B*) is associated with reduced binding of nuclear proteins *in vitro* and reduced activity of CYP3A4 *in vivo* (Rodriguez-Antona et al., 2005). The c.566T>C variation (*CYP3A4\*17*) is associated with decreased biotransformation of testosterone and chlorpyrifos *in vitro* (Dai et al., 2001) whereas the c.666T>C variation (*CYP3A4\*2*) is associated with reduced biotransformation of nifedipine *in vitro* (Sata et al., 2000).

CYP3A5 is also expressed both in the liver and the intestine. In contrast to CYP3A4, CYP3A5 is polymorphically expressed with readily detectable expression in about 10–20% of Caucasians, 30% of Japanese and 50% of African-Americans (Lamba et al., 2002). In the individuals with detectable CYP3A5 expression, it is CYP3A5, rather than CYP3A4 that constitutes the major part of total CYP3A expression (Lamba et al., 2002). The *CYP3A5* g.6986A>G variant (*CYP3A5\*3*) confers low or undetectable CYP3A5 expression. On the other hand, individuals carrying at least one copy of the g.6986A allele (*CYP3A5\*1*) express CYP3A5 protein (Hustert et al., 2001; Kuehl et al., 2001). The *NR1I2* g.-205\_-200delGAGAAG deletion variant in the *NR1I2* gene has been associated with increased expression of CYP3A4 in the liver (Lamba et al., 2006), and the g.-25385C>T, g.-24381 A>C, g.7635A>G, and g.8055C>T variants with a susceptibility to inflammatory bowel disease. These findings suggest that these SNPs are either functionally significant or in linkage disequilibrium with a functional variant (Dring et al., 2006).

### ***SLCO1B1* pharmacogenetics**

Large numbers of DNA sequence variations have been discovered in the *SLCO1B1* gene that affects the transport function of its expressed protein (Niemi, 2007). The most well characterized is the *SLCO1B1* c.521T>C variant. This variant is associated with reduced activity of OATP1B1 *in vitro* (Tirona et al., 2001) and increased plasma concentrations of simvastatin acid (Pasanen et al., 2006), atorvastatin (Pasanen et al., 2007), pravastatin (Niemi et al., 2004), rosuvastatin (Niemi et al., 2004) and repaglinide (Kalliokoski et al., 2008).

### ***ABCB1* and *ABCC2* pharmacogenetics**

Several DNA sequence variations have been identified in the *ABCB1* gene, including the c.1199G>A, the c.2677G>A/T, and the c.3435C>T variants. The *ABCB1* c.1199G>A variant has been associated with reduced transport activity of MDR1 *in vitro* (Woodahl et al., 2004). On the other

hand, the c.2677G>T variant has been associated with increased activity *in vitro* (Kim et al., 2001), and its c.2677G>A variant with reduced plasma concentrations of fexofenadine *in vivo* (Yi et al., 2004). The most studied SNP is the c.3435C>T variant which has been associated with reduced intestinal expression of MDR1 and increased plasma concentrations of digoxin *in vivo* (Hoffmeyer et al., 2000). In contrast to the above mentioned studies, for all these listed SNPs there are also discordant results which have been presented (Chinn and Kroetz, 2007; Leschziner et al., 2007). For instance, several studies reported that the c.3435C>T SNP is associated with an increased digoxin exposure, which suggests decreased MDR1 function in the intestine (Sakaeda et al., 2003). However, other studies reported described decreased exposure in c.3435C>T carriers, associated with an increased MDR1 function (Sakaeda et al., 2003). As the synonymous c.3435C>T variant has no effect on protein sequence, the c.3435T allele has been shown to have functional consequences only in haplotypes including the c.1236T or the c.2677T allele, or both (Kimchi-Sarfaty et al., 2007). Therefore, haplotypes, rather than a single genotype, may be important in study design and may clarify the role of *ABCB1* polymorphisms in drug pharmacokinetics.

Although the *ABCC2* polymorphisms are less well characterized than the *ABCB1* polymorphisms, the *ABCC2* c.24C>T variant has been associated with reduced expression of MRP2 in the kidney cortex (Haenisch et al., 2006). In contrast, the c.1446C>G variant results in an increased expression of MRP2 in the liver and reduced plasma concentrations of pravastatin (Niemi et al., 2006).

### 3. NON-GENETIC FACTORS THAT CAUSE VARIABILITY IN PHARMACOKINETICS

Age, body weight, disease, concomitant medication, and other environmental factors contribute to the inter-individual variability in absorption, distribution, metabolism and elimination of drugs. For instance, concomitant medication can inhibit or induce drug transporters or drug metabolizing enzymes and cause an increase or decrease in the absorption or elimination of a drug. As with concomitant medication, food is a complex mixture of chemicals and can interfere especially with the absorption process of many drugs, particularly dietary fat, which can slow gastric emptying. An additional important factor that explains the variability between patients is noncompliance, which can significantly contribute to the variability in drug response. In addition, the pharmacodynamic

responses of individuals are variable, making the concept of "one-dose-fits-all" often impractical (Rowland and Tozer, 1995).

### **Liver disease**

Disorders of the liver are a heterogeneous group of diseases and their effect on drug pharmacokinetics can be manifold. Hepatic disease can alter the clearance, oral bioavailability, and volume of distribution of drugs (Rowland and Tozer, 1995). Volume of distribution can be affected due to reduced hepatic protein synthesis, which can lead to decreased drug protein binding, edema, ascites, and an increase in the apparent volume of distribution. Hepatic clearance can decrease, if the blood flow to the liver hepatocytes is compromised (extra hepatic or intrahepatic shunting). This radically affects the clearance of drugs that have high hepatic extraction properties. Similarly, if the amount of metabolizing parenchyma is decreased, hepatic drug metabolism is adversely affected. The classification of hepatic insufficiency is difficult, but in general, low albumin, low pre-albumin, elevated clotting time, and the presence of encephalopathies signify that hepatic drug metabolism is significantly decreased (Verbeeck, 2008).

### **Kidney disease**

The effect of kidney disease on drug elimination can be estimated by measuring the glomerular filtration rate. An estimate of the glomerular filtration rate can be obtained when serum creatinine, body weight, age, and sex are known, and factored in the Cockcroft–Gault equation (Cockcroft and Gault, 1976). However, in children, changes in the glomerular filtration rate can be better estimated by measuring serum creatinine or cystatin-c and height and by using the Schwartz equation (Schwartz et al., 1987; Schwartz and Furth, 2007). Although commonly used, the equations that estimate glomerular filtration rate based on serum creatinine tend to overestimate the glomerular filtration rate and have a low sensitivity for renal dysfunction detection. The gold standard used to measure glomerular filtration rate is the renal inulin clearance or the plasma clearance of  $^{51}\text{Cr}$ -EDTA which is more precise. However, it is a cumbersome method to use in clinical practice (Garnett et al., 1967; Shemesh et al., 1985). After glomerular filtration, the renal tubules determine the ultimate composition of the urine. Kidney disease that affects tubular reabsorption or secretion can cause a wide variety of abnormal electrolyte profiles and lead to severe disorders related to fluid, electrolyte and/or acid-base

balance (Reidenberg and Drayer, 1980). In patients with renal insufficiency (uremia), creatinine clearance is decreased and the urinary excretion of drugs is diminished.

In addition to the urinary excretion, renal insufficiency and especially end-stage renal disease can also affect the non-renal clearance of many drugs by decreasing the expression of MDR1 and MRP transporters and CYP3A enzymes in the intestine (Nolin et al., 2008). Similar down regulation also occurs in the liver for CYP3A and for OATP1B1. Conversely, MDR1 in the livers of uremic subjects seems to be up-regulated (Nolin et al., 2008). Moreover, uremia can reduce the binding of drugs to blood and tissue components, possibly by increasing the unbound fraction of drugs and hence the volume of distribution and clearance (Reidenberg and Drayer, 1980). In addition, the absorption process of drugs could be adversely affected in chronic renal failure as the gastric emptying can become slower (Freeman et al., 1985; Kang, 1993).

### **Developmental changes in absorption, protein binding, and volume of distribution**

After birth, changes in pharmacokinetics occur as a consequence of changes in body composition, organ maturation, and the ontogeny of drug eliminating pathways. The most dramatic changes in drug absorption seem to occur in neonates: the rate of absorption of drugs is slower than that in older children (Kearns et al., 2003). However, oral medication for neonates is not common and in older children drug absorption seems to be either similar to those of adult absorption values or has even been described as being even faster than that found in adults (Rowland and Tozer, 1995). An additional factor to be considered in the very young children is their frequent feeding on milk. It is often impossible to prevent an interaction between a drug and food in infants and these effects can have a significant effect on the bioavailability of a drug (Bartelink et al., 2006).

In young infants the total body water content is high at around 80% of total body weight (Hartnoll et al., 1995) and the fat content is low (10–15%). The proportion of body water decreases to 55–60% by adolescence (Strolin Benedetti and Baltes, 2003; Bartelink et al., 2006). The percentage of body fat rises in the first year of life but decreases again during childhood. Only after the puberty induced changes in height, weight, lean body mass, and body fat content are complete; adult body composition is attained. Sexually mature females generally have about ten, percentage units, higher total body fat content than do males (Kennedy, 2008).



Body composition and plasma protein binding both affect the volume of distribution and half-life of drugs. In neonates and infants the concentration of total plasma proteins is about 15% less than in adults (Ehrnebo et al., 1971). Moreover, the concentration of albumin reaches adult levels as early as after the first year of life (Ehrnebo et al., 1971). In contrast, the hematocrit is high at birth and decreases rapidly in neonates. The hematocrit remains low in infants and children then increases to adult values in puberty (Behrman et al., 2000). The body composition dependent changes in volume of distribution are greatest because of the change in extracellular fluid volumes for hydrophilic drugs such as: panipenem, gentamycin, and linezolid. For such drugs the volume of distribution is significantly larger in neonates than in adults (Bartelink et al., 2006). Generally, the changes are smaller for lipophilic drugs and the influence of development on the apparent volume of distribution is not as clear as for hydrophilic drugs. This is because lipophilic drugs are largely distributed in tissues (Kearns et al., 2003).

#### **Developmental changes in renal elimination and drug transport**

The glomerular filtration rate ranges from about 2 to 4 ml/min/1.73 m<sup>2</sup> in neonates, increasing rapidly in the first weeks after birth. Thereafter the clearance rises steadily until adult values of about 80–130 ml/min/1.73 m<sup>2</sup> are reached at the age of one to two years (Kearns et al., 2003). Although very little is currently known about the ontogeny of kidney drug transporters, MDR1 and MRP2 are likely to attain their adult function after the first year(s) of life. This is supported by the fact that tubular secretion is immature at birth and reaches adult capacity during the first one to two years of life (Kearns et al., 2003).

#### **Developmental changes in hepatic and intestinal metabolism and drug transport**

Obtaining representative tissue samples has been one of the major obstacles in studying the ontogeny of drug metabolizing enzymes and drug transporters. As far as the pharmacological implications are concerned, the information on developmental changes in enzyme activity as expressed as per gram of liver is more relevant than the developmental variation of messenger RNA (mRNA) concentrations. Messenger RNA concentrations are sometimes measured when the tissue samples obtained are too small for protein content and activity analyses. According to the existing studies, distinct patterns of isoform-specific developmental expression of CYPs have been observed postnatally (de Wildt et al., 1999). The largest changes seem to occur in the first years of

life, whereas the hormonal changes of puberty have not been shown to significantly affect drug metabolizing enzyme activity (Kennedy, 2008).

Before birth, CYP3A7 activity in the liver (per gram of liver) dominates and then declines rapidly thereafter. In contrast, CYP3A4 activity is very low at birth and increases close to adult levels in the first year of life (Lacroix et al., 1997; Treluyer et al., 1997; de Wildt et al., 1999; Hines, 2008). Conversely, according to a study on 59 adult human liver samples, significant CYP3A7 protein expression was found in 10% of the patients which contributed to 10–40% of total CYP3A levels in these livers (Sim et al., 2005).

The ontogeny of CYP3A5 is less well characterized. In small scale studies the expression of CYP3A5 was found in both fetal and pediatric liver samples, but with highly variable levels and independent of age (Stevens et al., 2003; Hines, 2008). The ontogeny of MDR1, MRP2 and OATP1B1 in the liver is currently uncharacterized.

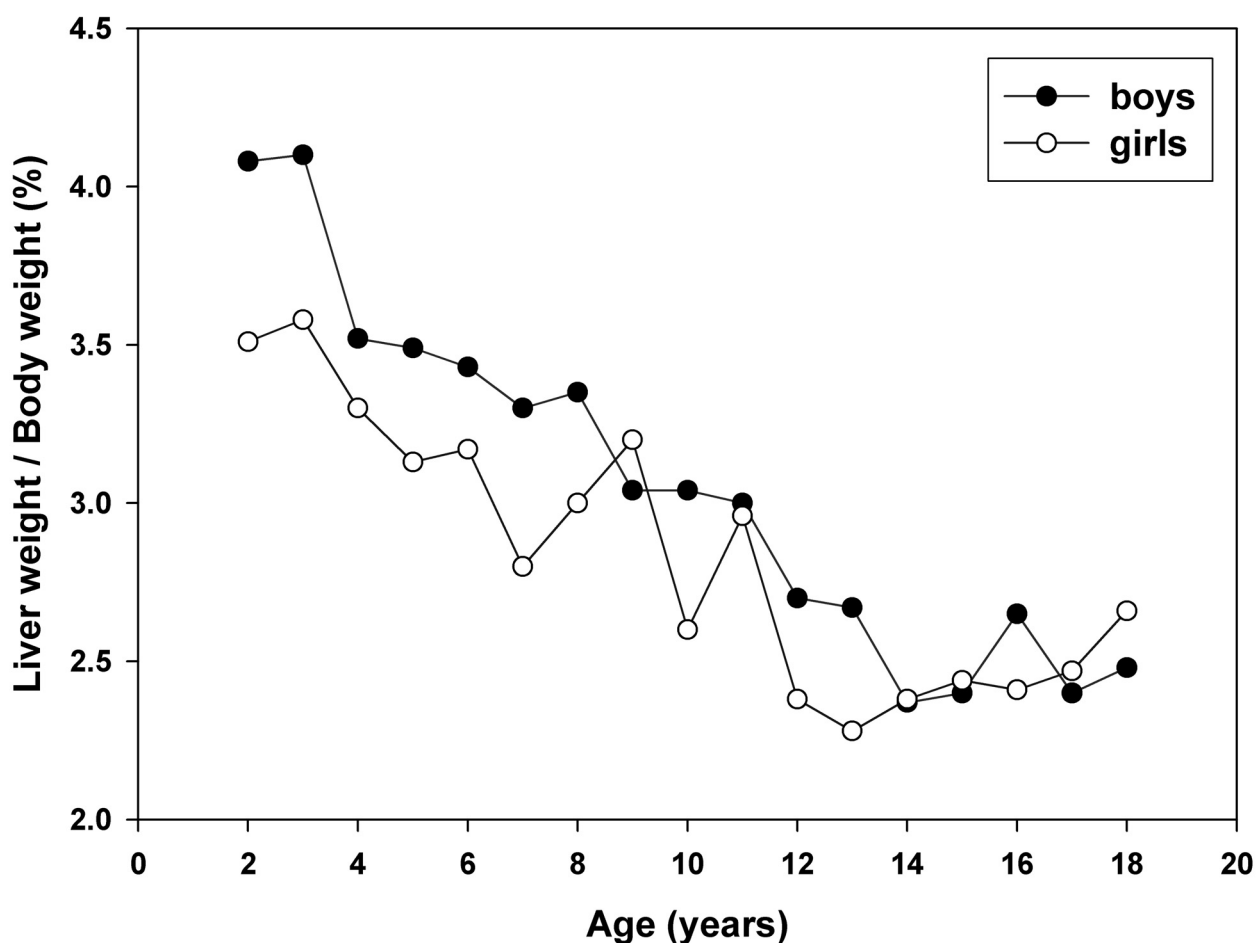
In one study, the CYP3A4 activity was close to non-existent in the fetal duodenum then increased, but with high variability, in infants and small children (Johnson et al., 2001). In another study the intestinal CYP3A4 mRNA levels were 2-fold higher in neonates compared with fetuses. Furthermore, young adults had four to five times higher levels of mRNA than did neonates (Miki et al., 2005). However, another study reported that a decrease in CYP3A4 and CYP3A5 mRNA levels in children aged between one to six years old was found when compared to the corresponding expression levels in infants aged 1–12 months (Fakhoury et al., 2005). The authors of this study suggested that post-transcriptional regulatory mechanisms may be involved in the expression of the actual CYP3A enzyme (Fakhoury et al., 2005). The same authors detected significant levels of MDR1 mRNA in the small intestine across the whole pediatric age-range, without any specific developmental pattern. Nevertheless, Miki et al. found slightly higher expressions of intestinal MDR1 in neonates than in fetuses and an approximately 4-fold higher expression in young adults compared to neonates (Miki et al., 2005). Currently no studies have been published where the ontogeny of MRP2 has been studied in the intestine.

**Sex-related changes in drug exposure**

Differences in metabolic ratio attributable to sex are generally small. According to some studies, males have a higher activity relative to females for CYP1A2, whereas the activity of CYP3A4 has been slightly lower in men than in women (Scandlyn et al., 2008). However, other studies have found no sex-related differences in CYP1A2 or CYP3A4 activity (Fahr, 1993; Bebia et al., 2004; Backman et al., 2008; Greenblatt and von Moltke, 2008). The majority of published reports have found minimal or no sex-related differences in hepatic or intestinal CYP3A expression/activity (Greenblatt and von Moltke, 2008). Other factors that could explain sex-related pharmacokinetic differences include the lower body weight and organ size, lower hepatic blood flow, higher percentage of body fat affecting volume of distribution, and lower glomerular filtration rate found in women than in men. Furthermore, the effects of sex on CYP1A2 or CYP3A4 activities are largely outweighed by the wide inter-individual variability in CYP1A2 or CYP3A4 activity due to other factors than sex. In conclusion, the results of the above mentioned studies suggest that gender explains only a small proportion, if any, of the individual variation in CYP1A2 or CYP3A phenotypes, and that male/female differences are unlikely to be of clinical importance.

**Body size -related changes in pharmacokinetics**

A consistent observation in clinical studies of drugs metabolized in the livers of young children below the age of ten years is a higher body weight-adjusted clearance compared to that found for adults. This finding necessitates relatively higher weight-based dose requirements in pre-teen patients (Kearns et al., 2003). Possible reasons for the high body weight-adjusted clearance in children include a larger liver volume adjusted to body weight (Murry et al., 1995; Takahashi et al., 2000; Johnson, 2003; Strolin Benedetti and Baltes, 2003), or a high concentration of catalytically active CYPs. However, evidence for the latter hypothesis is scarce (Blanco et al., 2000) and the liver enzyme activity seems generally to be constant from childhood to adulthood (Bartelink et al., 2006). Therefore, developmental changes in clearance are largely due to age-dependent changes in relative liver size (Figure 1).

**Figure 1. Mean liver weight as a percentage of body weight in healthy boys and girls.**

The mean liver weight data and the percentiles of body weight were obtained from (Behrman et al., 2000; Stocker and Dehner, 2001; Bond, 2004).

Accordingly, after the liver enzymes and transporters have matured, the rate of metabolism is mainly dependent on liver size. For CYP3A4 this takes about one year. Thus, liver volume, blood flow and biliary excretion correlate well with an estimate of body size such as, the body surface area (BSA). The use of surface area or body weight as a scaling factor for size is common in pediatrics. However, pharmacokinetic parameters, adjusted to body weight, may vary as a function of age. Using body surface area as a standardization factor would be more accurate, but the need for both height, weight, and an equation makes its use more cumbersome than just the body weight alone. Moreover, the exact equation used can cause additional problems. For instance, the most common equation used to calculate BSA (Du Bois and Du Bois, 1916) was based on nine subjects only. Therefore, when calculating pediatric BSA, the Gehan-George equation

based on a population of 401 patients, including children, should be used in preference (Gehan and George, 1970).

An allometric model has been demonstrated to be an even better approach to standardize the effect of body size on clearance and volume of distribution. Allometrics formulates models for relating body function and morphology to that of body size (Holford, 1996). According to allometric principles for estimating the metabolic rate of the body, i.e. clearance (CL), the following equation is used  $CL = CL_{\text{standard}} \times (\text{individuals weight/standard weight})^{3/4}$ , where the standard weight is the normal weight of a given population, for example 70 kg in healthy adults. In order to estimate the volume of distribution (Vd) the following equation is used  $Vd = Vd_{\text{standard}} \times (\text{Individuals weight/standard weight})^1$  (West et al., 1997). The  $3/4$  exponent was derived from a general model that describes how essential materials are transported through space-filled fractal networks of branching tubes (West et al., 1999). Allometric scaling has been successfully applied in inter-species scaling (Holford, 1996) and in the pharmacokinetic modeling of substrates that undergo liver metabolism, such as paracetamol (Anderson et al., 2000), ciprofloxacin (Rajagopalan and Gastonguay, 2003), zidovudine (Capparelli et al., 2003), midazolam (Björkman, 2006), and alfentanil (Björkman, 2006). Although the above allometric weight model has been highly useful in children and adults, it is poor at predicting changes in clearance and volume of distribution in the earliest period of life after birth. In neonates and children under the age of one or two years, the maturing enzyme systems play a large role in the total variability not attributable to patient size (Johnson, 2005).

#### 4. THERAPEUTIC USE OF CYCLOSPORINE

##### **The discovery of cyclosporine and its early clinical use**

After transplantation of foreign tissue into the human body, the T-lymphocytes of the recipient recognize the non-self proteins and peptides. Without immunosuppression this leads to a cascade of immunological reactions resulting in the rejection of the allograft. The earliest attempts to control the immune responses were with total body irradiation and later with azathioprine, glucocorticoids, and anti-lymphocyte serum. However, the 1-year allograft survival rates were generally below 60% in kidney transplantation, and mortality that was primarily attributed to infection was over 15% (Kahan, 1983). Moreover, renal replacement therapy remained controversial in pediatric patients until the early 1980s due to the large number of complications and the severe growth impairment in children resulting from the use of large doses of glucocorticoids, (Chantler, 1979; Cooney et al., 1997).

In 1970 cyclosporine, a lipophilic cyclic polypeptide, was extracted from the fungus *Tolypocladium inflatum* Gams (Petcher et al., 1976). In 1972, Borel et al. discovered its immunosuppressive properties in rodents and the first clinical trials in humans were conducted in 1974 by Calne et al. (Borel, 1983). The major finding was that cyclosporine was more selective in its immunosuppressive properties than were azathioprine and the glucocorticoids. Cyclosporine reversibly inhibits T-cell mediated alloimmune and autoimmune responses (Borel, 1976). More specifically, cyclosporine inhibits the cell signal mediator calcineurin (Liu et al., 1991), a protein phosphatase, and thereby interferes with the synthesis of a variety of lymphokine mediators, particularly interleukin-2 (Yoshimura and Kahan, 1986), which is critical for T-cell proliferation and maturation.

Before cyclosporine was discovered immunosuppressive medication consisted mainly of azathioprine and glucocorticoids (Kahan, 1983) and with this drug combination both the acute rejection frequency and the rate of infections remained high (Murray et al., 1962; Hume et al., 1966). After the introduction of cyclosporine to the treatment protocols, the allograft survival rates increased from 50% to 80% in a one year period (Lancet, 1983; N Engl J Med, 1983) and to 60–70% at five years follow-up (Merion et al., 1984; Calne, 1987). However, despite the selective inhibition of T-cell mediated immune responses by cyclosporine, its use was not without problems.

In the early trials the mortality rates were high due to infection and lymphoma. Fortunately, the initial problems of infection and malignancy were largely overcome by reduction of the dosage of cyclosporine and cyclosporine use became the standard care in the beginning of the 1980s (Kahan et al., 1985; Najarian et al., 1985). However, it was soon discovered that cyclosporine is nephrotoxic and has a narrow therapeutic range. Moreover, a large variability to cyclosporine exposure between patients was noted (Kahan, 1989a). Therefore, to reduce the number of patients with insufficient or excessive cyclosporine concentrations, a target concentration monitoring approach was adopted (Kahan et al., 1985; Rogerson et al., 1986).

### **Indications for cyclosporine treatment**

In addition to renal transplantation, cyclosporine has successfully been used in other solid organ transplantation as prophylaxis for graft rejection, and as prophylaxis against graft-versus-host-disease in bone marrow transplantation. Cyclosporine is also widely used for the treatment of auto-immune diseases including psoriasis, rheumatoid arthritis, endogenous uveitis, nephrotic syndrome, severe ulcerative colitis, severe atopic dermatitis, and severe keratoconjunctivitis sicca (Dunn et al., 2001).

### **Adverse effects of cyclosporine**

Many of the serious adverse effects caused by cyclosporine are exposure related. However, the exact exposure-effect relationship has not been established and the pharmacodynamic inter-individual variability is large, as some patients experience toxic effects even at low cyclosporine concentrations. The most serious adverse effects include acute and chronic nephrotoxicity, hypertension, and neurotoxicity. All of these occur with a frequency of >10%. Acute nephrotoxicity is characterized by acute vasoconstriction of the kidney arterioles and arteries, which usually presents as a reversible decrease in glomerular filtration rate. Acute nephrotoxicity is reported to affect between 25% to 40% of kidney, heart or liver transplant recipients being treated with cyclosporine (Rossi et al., 1993). In addition to the acute nephrotoxicity, the long-term use of cyclosporine induces interstitial fibrosis, tubular atrophy, and vascular lesions, particularly fibrous thickening of the arterial intima in the kidneys. However, these morphological lesions cannot be attributed solely to cyclosporine toxicity. In addition to chronic cyclosporine toxicity, many other immunologic and non-immunologic factors contribute to the development of chronic allograft nephropathy (Mihatsch et al., 1995; Tönshoff and Höcker, 2006).

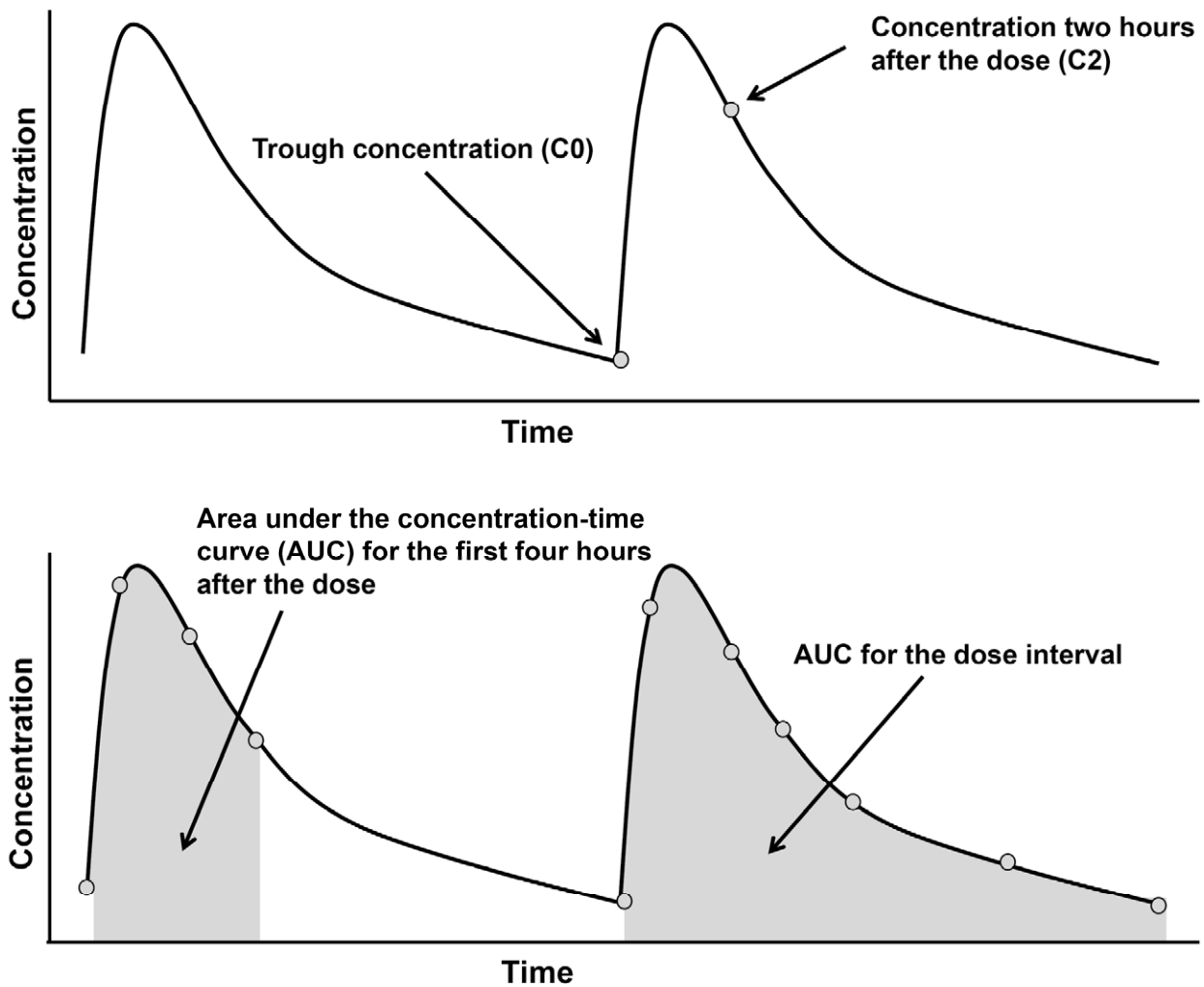
Mild to moderate hypertension has been documented in up to 50% of renal transplant patients receiving cyclosporine. Neurological symptoms, including headaches and tremor have been documented to occur with a frequency over 10%. However, paresthesias are less common and they usually occur with a frequency of less than 10% (Rossi et al., 1993). Other common side effects that usually occur with a frequency <10% include hyperlipidemia, anorexia, nausea, hyperurichemia, hyperkalemia, hypomagnesemia, gingival hyperplasia, and hypertrichosis (Dunn et al., 2001).

### **The evolving therapeutic drug monitoring of cyclosporine**

Early studies showed that patients with low cyclosporine trough levels experienced acute rejection with an increasing frequency whereas those with high trough levels had renal dysfunction and other toxicities (Kahan et al., 1984; Kahan et al., 1985; Rogerson et al., 1986). Although trough-level (C<sub>0</sub>) monitoring reduced the number of patients with excessive or insufficient concentrations, the area under the concentration-time curve (AUC) was found to be a more precise predictor of incidence of acute rejection (Lindholm and Kahan, 1993; Schroeder et al., 1995). However, the measurement of AUC is time-consuming, expensive, and requires repeated blood sampling, therefore surrogate monitoring strategies that correlate with the total AUC, have been developed. These include: limited AUC sampling monitoring; absorption phase monitoring (AUC<sub>0-4</sub>) and two hours after dosing, (C<sub>2</sub>) monitoring (Kahan, 2001). The most commonly used monitoring strategies are C<sub>0</sub> and C<sub>2</sub>, although there is ongoing debate as to which monitoring method would be the best to use (Knight and Morris, 2007).



**Figure 2. The most commonly applied therapeutic drug monitoring methods for cyclosporine.**



The curves depict typical cyclosporine concentrations for cyclosporine that is administered twice daily.

Regardless of the exact monitoring strategy, the use of cyclosporine with other potent immunosuppressive drugs in renal transplantation has resulted in a dramatic decrease in acute rejection rates and an excellent short-term patient and graft survival in adults and children (Meier-Kriesche et al., 2004; Tönshoff and Höcker, 2006). Unfortunately, long-term graft survival rates have not similarly increased - chronic allograft nephropathy is the main reason for graft loss in children after the first post-transplant year (Tönshoff and Höcker, 2006). The nephrotoxic effects of the calcineurin inhibitors, cyclosporine and tacrolimus, together with individual immunologic and non-immunologic factors contribute to the development of chronic allograft nephropathy (Tantravahi et al., 2007; Srinivas and Meier-Kriesche, 2008). In addition, the use of potent immunosuppression significantly increases the risk for developing malignancies of the skin,

gynecologic organs, the rectum, and especially in children, post-transplant lymphoproliferative disorder, (Buell et al., 2006; McDonald et al., 2008). In the pediatric renal transplant population the incidence of post-transplant lymphoproliferative disorder has ranged from 1% to 4.5% (Dharnidharka et al., 2001; Funch et al., 2002). Many transplantation centers have undertaken the approach of minimizing immunosuppression to reduce the adverse effects of immunosuppressive medication (Tönshoff and Höcker, 2006; Matas, 2007; Srinivas and Meier-Kriesche, 2008). However, because the optimal immunosuppressive requirements for each individual patient are unknown, all minimization strategies have the potential risk for under-immunosuppression, which can result in acute rejection and premature graft loss (Srinivas and Meier-Kriesche, 2008).

The greatest problem with cyclosporine concentration controlled therapeutic drug monitoring is that even a strict adherence to recommended drug levels cannot guarantee freedom from rejection or toxicity. Transplant biopsies still serve as an important tool for the clinician to monitor the immunological status of the graft. Thus, a non-invasive method for systemic immune monitoring of the transplant recipient would be of benefit. Cyclosporine inhibits phosphatase calcineurin (Liu et al., 1991), thus one strategy is to measure the calcineurin phosphatase activity in circulating blood leucocytes as a prelude as to whether or not to adjust the cyclosporine dosing. In effect, the inhibition of calcineurin by cyclosporine is rapidly reversible and closely correlated with cyclosporine blood concentrations throughout the dosing interval (Batiuk et al., 1995; Halloran et al., 1999). However, this strategy measures only the effects of cyclosporine, and newer assays can directly measure the immune function of T-cells, and help clinicians to discern between immune profiles of over and under-immunosuppression (Gerrits et al., 2007). Furthermore, the measured effects of these assays show large inter- and intra-individual variability, thus histopathological analysis of the transplant remains the gold standard of rejection classification and cyclosporine toxicity (Gerrits et al., 2007; Israeli et al., 2007; Yano, 2008).

A contributory factor to the large inter-individual variability in cyclosporine pharmacodynamics is patient age. In an in vitro study, the peripheral blood monocytes of cyclosporine treated infants (age < 1 year, n=10) showed a two-fold lower monocyte proliferation rate and a seven-fold lower interleukin-2 expression rate than did older children and adults (n=40) with similar cyclosporine concentrations (Marshall and Kearns, 1999). In conclusion, the optimal target concentration levels and optimal monitoring strategies for cyclosporine remain elusive especially after the first year

post-transplantation (Knight and Morris, 2007). However, for the time being, concentration controlled cyclosporine therapeutic monitoring either at C<sub>0</sub>, C<sub>2</sub>, or with an abbreviated AUC, seems to be the best parameters to use for individualizing cyclosporine dosing in adults and children (Dunn, 2003; Kahan, 2004).

## **5. CYCLOSPORINE PHARMACOKINETICS**

### **Cyclosporine formulations**

Oral cyclosporine is available in two dosage forms: the oil-based formulation (Sandimmune) and the newer microemulsion formulation (Sandimmune Neoral). The oil-based formulation and the microemulsion formulation are both available as an oral solution or as gelatine capsules. In Finland, only the microemulsion oral dosage form is available at the moment (Novartis, 2007). According to the manufacturer the microemulsion oral solution and gelatinized capsule formulations of cyclosporine are bioequivalent (Novartis, 2007). The intravenous formulation of cyclosporine consists in the form of cyclosporine, polyoxyethylated castor oil (Cremophor EL) and of alcohol (Novartis, 2007). Approximately 1 in 1000 patients who have received intravenous cyclosporine has experienced anaphylactoid reactions. Although the exact cause of these reactions is unknown, it is believed to be due to the polyoxyethylated castor oil used as the vehicle for the intravenous formulation. These reactions include: angioedema, flushing of the face and upper thorax, with acute respiratory distress, dyspnea, wheezing, blood pressure changes, and tachycardia (Novartis, 2007).

### **Cyclosporine assay methods**

Cyclosporine concentrations are most commonly measured in the whole blood (Kivistö, 1992). Although cyclosporine concentrations could also be measured in plasma, the fact that blood temperature does not affect the whole blood cyclosporine concentrations, as opposed to the plasma concentrations, makes the use of whole blood for the detection of cyclosporine easier (Sketris et al., 1995). At room temperature, about 70% of cyclosporine is distributed in blood cells and the remaining 30% is found in plasma, largely bound to lipoproteins, in a blood sample containing 500 µg/L of cyclosporine (Akhlaghi and Trull, 2002). According to an early study, the measurement of cyclosporine in whole blood allowed the best distinction between patients who respond favorably or less favorably to cyclosporine treatment (Lindholm et al., 1990a). Although

cumbersome, it is also possible to measure the unbound concentration of cyclosporine in plasma. In a study on 66 patients both the whole blood cyclosporine concentrations and the free fraction of cyclosporine were measured for a period of six months after transplantation. The aim of the study was to determine cyclosporine concentrations that best predict acute rejection. However, in this study the measurement of unbound cyclosporine concentrations did not provide any additional information that would help clinicians avoid rejection. Thus, measuring the free fraction of cyclosporine was not recommended as the method is laborious and complicated (Lindholm, 1991b).

The reference technique for the measurement of cyclosporine concentrations in blood is high pressure liquid chromatography (HPLC), particularly when coupled with mass-spectrometry (Holt et al., 2000). However, HPLC assays can specifically quantify cyclosporine concentrations in the blood, though the use of antibody-based assays is often cheaper and easier to apply. Specific monoclonal antibody-based assays include the radioimmunoassay (RIA), the fluorescence polarization immunoassay (FPIA), the enzyme multiplied immunoassay (EMIT), the cloned enzyme donor immunoassay (CEDIA), and the chemiluminescent microparticle immunoassay (CMIA) (Andrews and Cramb, 2002). Although these immunoassay techniques are claimed to be specific for cyclosporine, they all tend to overestimate cyclosporine blood concentrations by also measuring cyclosporine metabolites to varying degrees (Kivistö, 1992; Andrews and Cramb, 2002). Moreover, the metabolite/parent compound ratio of the immunoassays changes with time in the blood and can also vary with time after transplantation (Johnston and Holt, 1999). In general, the antibody-based assays produce about 10%–30% larger concentration values than does HPLC (Hirvisalo et al., 1990; Johnston and Holt, 1999; Safarcík et al., 2001; Johnston et al., 2003; Rodriguez et al., 2005). More specifically, the RIA method has been shown to give 10%–40% larger concentrations than HPLC (Hirvisalo et al., 1990; Johnston and Holt, 1999; Safarcík et al., 2001). The FPIA assay has produced even higher values than the RIA method. These varied from about 20% greater values at C0 and 10% greater values at C2 (Andrews and Cramb, 2002; Johnston et al., 2003; Rodriguez et al., 2005). On the other hand, the newer CMIA method has determined concentrations in the same range as the RIA method for cyclosporine levels lower than 300 µg/L. However, for cyclosporine concentrations higher than 300 µg/L, the CMIA assay can determine 10–30% larger values than the RIA method (HUSLAB laboratory manual, [www.huslab.fi](http://www.huslab.fi)).

### Cyclosporine absorption

The absorption of the oil-based formulation of cyclosporine from the duodenum and jejunum is bile-dependent (Friman and Backman, 1996). The emulsification of the cyclosporine in oil mixture with bile salts is necessary before this cyclosporine formulation can be absorbed. Therefore, it has been hypothesized that it is the disturbances in the emulsification process that causes the poor and variable absorption of the oil-based cyclosporine formulation observed. Furthermore, poor absorption from the gastrointestinal mucosa used to explain the variable oral bioavailability of cyclosporine when the drug is given in an oil solution formulation (Friman and Backman, 1996). The absolute oral bioavailability of the oil-based formulation was on average 30%, the values range between 10% and 80% in adults (Friman and Backman, 1996). The mean time to reach maximum concentration ( $T_{max}$ ) varied, on an average from two to four hours (range 1–8 hours) (Ptachcinski et al., 1985a; Ptachcinski et al., 1985b; Awni et al., 1989; Lindholm et al., 1990b).

The microemulsion formulation of cyclosporine has self-emulsifying properties and creates micelles in the stomach that are absorbed by the small intestine with less need for the presence of bile. Therefore, compared to the oil-based formulation, the bioavailability of the microemulsion formulation is generally about 30% higher with an absolute bioavailability of about 40% (Holt et al., 1994). Moreover, the  $T_{max}$  occurs about one hour earlier than with the oil-based formulation, i.e. 1–2 hours after the dose (Holt et al., 1994; Friman and Backman, 1996). In comparison, the microemulsified formulation seems to display less inter-occasion variability at: maximum cyclosporine concentration ( $C_{max}$ ),  $T_{max}$ , trough concentration, and AUC than does the oil-based formulation (Kahan et al., 1995). However, the inter-individual variability in cyclosporine pharmacokinetics for the microemulsion formulation seems to remain as wide as for the oil-based formulation (Kahan et al., 1995).

### Distribution of cyclosporine throughout the body

According to data obtained from adult studies the steady state volume of distribution during intravenous dosing has been reported to be 3–5 L/kg in solid organ transplant recipients (Ptachcinski et al., 1986; Lill et al., 2000). The large volume of distribution is consistent with the finding that cyclosporine is extensively bound to blood, plasma and tissue constituents (Akhlaghi and Trull, 2002). As much as 90% to 98% of the circulating cyclosporine is bound to red blood cells and plasma protein (Akhlaghi and Trull, 2002). In addition, cyclosporine widely distributes in

tissues and accumulates mainly in fat-rich organs such as the liver, adipose tissue, and lymph nodes. Large concentrations have also been found in the large intestine, breast tissue (and breast milk), stomach adrenal glands, esophagus, pancreas, spleen and kidney (Atkinson et al., 1982; Lensmeyer et al., 1991). The distribution of cyclosporine in blood is dependent on temperature, cyclosporine concentration, hematocrit and plasma lipoproteins (Akhlaghi and Trull, 2002). The percentage of cyclosporine in plasma has an inverse linear relationship to that of the hematocrit value (Rosano, 1985). Moreover, cyclosporine binds to plasma proteins with about 20% to 60% of the total cyclosporine amount, in plasma, being bound to lipoproteins (Lemaire and Tillement, 1982; Sgoutas et al., 1986). Higher concentrations of plasma lipids result in increased cyclosporine binding to VLDL, IDL and LDL, and decreased binding to the HDL fraction (Gardier et al., 1993). In organ transplant patients cyclosporine clearance was significantly less in patients with hyperlipidemia than in those with normal cholesterol levels (Awni et al., 1990). In another study the volume of distribution at steady-state was negatively correlated with the concentration of total cholesterol (Gardier et al., 1993). In addition to total cholesterol, hematocrit has also been demonstrated to affect cyclosporine clearance (Yee et al., 1988; Wu et al., 2005).

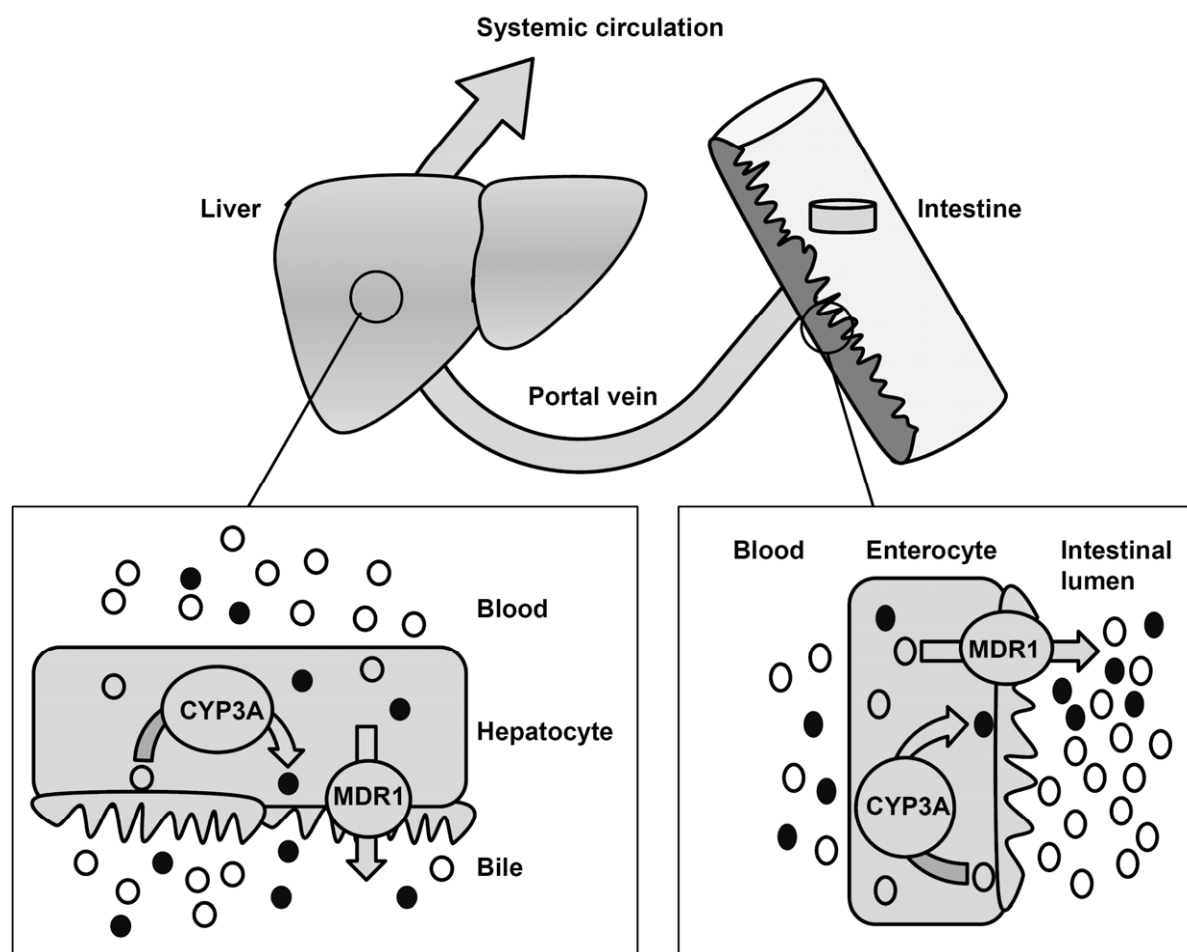
### **Evidence for pre-hepatic metabolism of cyclosporine**

Poor absorption by the gastrointestinal mucosa has been alleged to be the main cause for the variable and poor bioavailability of cyclosporine oil-based solution (Friman and Backman, 1996). However, to test whether the poor oral bioavailability of cyclosporine is attributable to poor absorption or extensive intestinal metabolism, cyclosporine was given intravenously and orally (oil-based formulation) with and without oral rifampin (Hebert et al., 1992), oral erythromycin (Gupta et al., 1989a) and oral ketokonazole (Gomez et al., 1995). These studies concluded that the drug interactions did not have an effect on the rate of the absorption, but that both the CYP3A inducer rifampin and the CYP3A4 inhibitors altered cyclosporine bioavailability to a greater extent than would be predicted from the changes in hepatic clearance alone. Moreover, the mean hepatic extraction ratio of cyclosporine was 0.25 (Wu et al., 1995). Thus, with an oral bioavailability of about 30%, the pre-hepatic extraction ratio for cyclosporine at 0.6 was approximately two times larger. These findings suggest that it is the extensive pre-hepatic metabolism rather than poor absorption that causes the low cyclosporine oral bioavailability, assuming that none of cyclosporine is excreted in unchanged form in the feces.

Other evidence supporting the extensive pre-hepatic metabolism of cyclosporine also exists. Hoppu et al. investigated the pharmacokinetics of cyclosporine in 20 children prior to renal transplantation and the cyclosporine blood concentrations were measured after intravenous and oral test doses by both specific and nonspecific monoclonal radioimmunoassays. The mean ratio of AUCs measured by nonspecific and specific RIA was 1.96 after oral administration and 1.43 after i.v. administration, which suggests pre-systemic, pre-hepatic metabolism of cyclosporine (Hoppu et al., 1991a). In an elegant experiment, Kolars et al. instilled cyclosporine into the small intestines of two liver transplant patients during the anhepatic phase of a liver transplant operation, and they subsequently measured cyclosporine metabolites in the blood, which was presumably due to intestinal metabolism (Kolars et al., 1991).

### **Cyclosporine metabolism and clearance**

Cyclosporine undergoes extensive metabolism in the liver and the small intestine and is converted into more than 30 metabolites by both phase I and II metabolism. The primary phase I metabolic reactions are catalyzed mainly by the CYP3A4 and CYP3A5 enzymes (Maurer, 1985; Bertault-Peres et al., 1987; Kronbach et al., 1988). Moreover, cyclosporine is both an inhibitor and a substrate of the MDR1 transporter (Goldberg et al., 1988), which is located in the enterocytes (apical surface) and the hepatocytes (baso-lateral surface) (Fromm, 2004). Enterocytes and hepatocytes both express CYP3A4 and the efflux transporter MDR1 (Watkins, 1997), which leads to an increased exposure of cyclosporine to metabolism by CYP3A4 through repeated cycles of absorption and efflux (Zhang and Benet, 2001).

**Figure 3. Cyclosporine metabolism occurs both in the intestinal wall and the liver.**

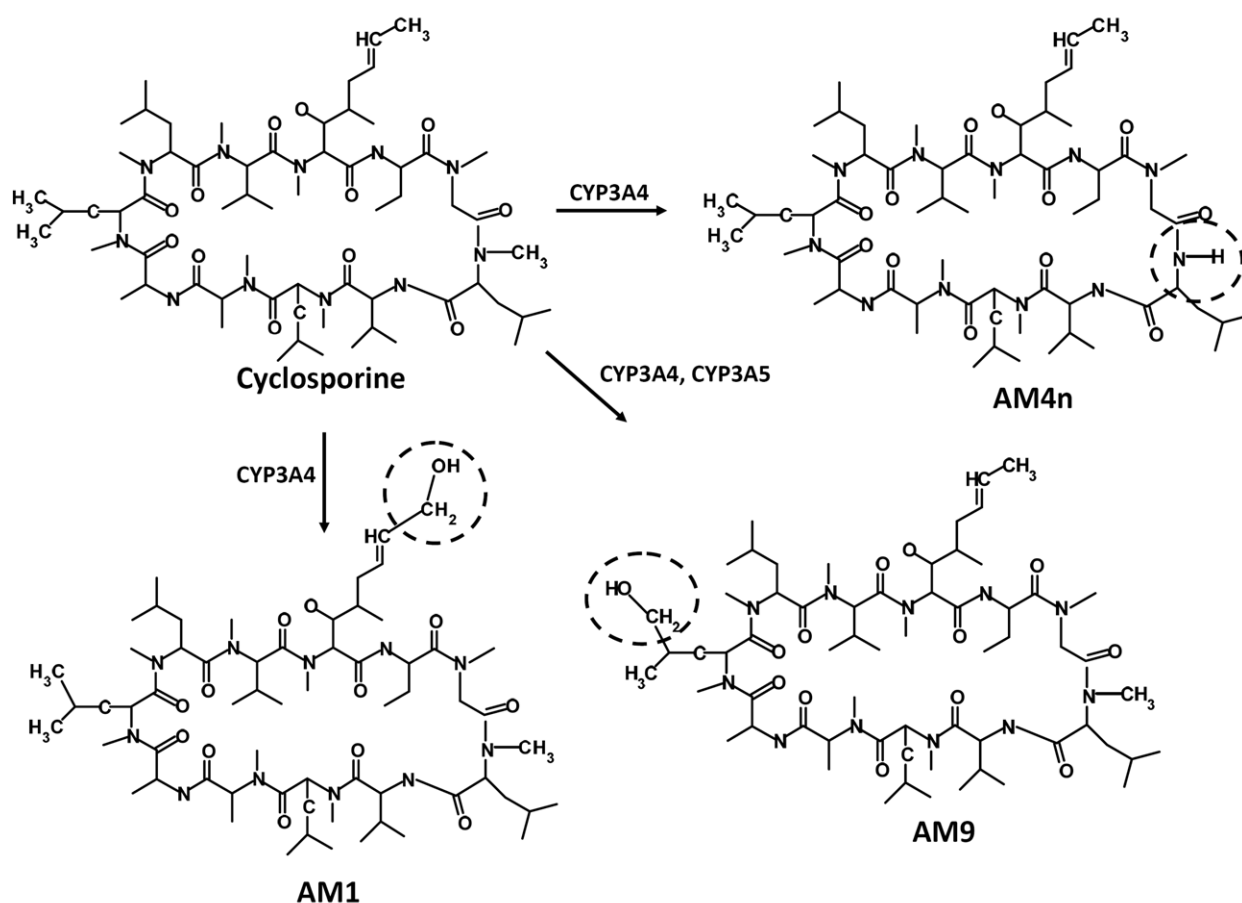
CYP3A and MDR1 are co-expressed in enterocytes and hepatocytes, leading to an increased exposure of cyclosporine to metabolism by CYP3A. The empty circles depict cyclosporine and the filled circles depict cyclosporine metabolites.

Cyclosporine metabolites have been specified by local code letters and (or) numeric designations based on their HPLC elution times in distinct mobile phases. However, the recommended cyclosporine metabolite nomenclature is based upon cyclosporine being A, followed by an M designating a metabolite, and a number indicating the amino acid position of the conversion. The use of two numbers indicates similar transformations at two different positions. Unless designated by an "n" to signify de-methylation or a "c" to signify cyclization, the metabolites represent hydroxylations. For example, the cyclosporine metabolite found in large quantities in humans, was previously designated as metabolite 17 (M17), but according to the recommended nomenclature that metabolite is now designated AM1, because the compound is derived from cyclosporine and carries an oxidation at the 1 $\eta$ (8') site (Kahan et al., 1990). Hydroxylated (AM1, AM9), de-



methyated (AM4n), and position-1-cyclized (AM1c) metabolites have been characterized. In all the discovered metabolites, the cyclic structure of cyclosporine is preserved (Christians and Sewing, 1993). The hydroxylated and de-methylated metabolites are found most abundantly in blood. After steady-state has been attained in cyclosporine dosing, the concentrations of cyclosporine metabolites approach the concentrations of the parent compound (Schwinghammer et al., 1991). Most of the absorbed cyclosporine (90%) is excreted in bile as metabolites, with less than 1% excreted as the unchanged drug. The rest is excreted in the urine, with less than 0.1% as the unchanged drug (Kelly and Kahan, 2002).

**Figure 4. The chemical structure and metabolic pathway of cyclosporine.**



AM1, AM9, and AM4n are the predominant metabolites found in human blood and urine. Other metabolites of cyclosporine result from further metabolism of the primary metabolites.

Cyclosporine blood clearance is approximately 0.3–0.5 L/h/kg in adults who have undergone solid organ transplantation (Ptachcinski et al., 1985a; Ptachcinski et al., 1986; Dunn et al., 2001). As the hepatic blood flow is approximately 1.2 L/h/kg (Rowland and Tozer, 1995) in healthy adults, cyclosporine is considered to have a low to medium hepatic extraction ratio ( $0.4 \text{ L/h/kg} / 1.2 \text{ L/h/kg} = 0.33$ ) (Rowland and Tozer, 1995). Therefore, cyclosporine clearance can be influenced by changes in its unbound fraction according to the well-stirred model (Pang and Rowland, 1977).

### **Immunosuppressive activity of cyclosporine metabolites**

The degree of the immunosuppressive activity attributable to cyclosporine metabolites in blood has been estimated to range from 10% to 30% of that of the parent compound (Ozbay et al., 2007). However, measuring immunosuppressive activity, depends on the *in vitro* assay used, which makes comparisons between the studies difficult (Karamperis et al., 2006). In a recent *ex-vivo* experimental study, cyclosporine and cyclosporine metabolites were added to the whole blood and calcineurin inhibition was measured. The authors concluded that the inhibition of calcineurin by each metabolite was less than 10% of that of cyclosporine (Karamperis et al., 2005). Assays that also measured cyclosporine metabolites correlated more strongly with calcineurin phosphatase inhibition than did cyclosporine-specific assays (Ozbay et al., 2007). This suggests that the commonly used immunoassays might have a clinical advantage over the more specific analytical methods for detecting cyclosporine concentrations.

### **Cyclosporine dose-linearity, half-life, and dosing frequency**

The dose-linearity of intravenous cyclosporine was studied in six renal transplant patients who received a 5 mg/kg infusion of cyclosporine over 3, 6, or 24 hours (Gupta et al., 1989b). Each patient received cyclosporine with the three infusion rates. The results showed that, neither the clearance nor the other disposition parameters changed with different infusion time, suggesting that cyclosporine elimination did not become saturated in the therapeutic concentration range. Similarly, cyclosporine oral administration (as the microemulsion formulation) produced dose-linear pharmacokinetics in the dose-range of 200 to 800 mg (single doses) (Müller et al., 1994b). In this study, 48 healthy male volunteers received single doses of cyclosporine in the range of 3–10 mg/kg. In a six-month randomized multicenter trial including 60 maintenance liver transplanted patients, cyclosporine pharmacokinetics was characterized on twice daily dosing and then switched to once daily dosing (Kovarik et al., 2008). Before switching the dosing frequency the

patients received about 1.1 mg/kg of cyclosporine twice daily and after the switching about 2.2 mg/kg once per day. The diurnal AUC ( $AUC_{0-24h}$ ) was about 30% higher when cyclosporine was received once daily than when it was received twice daily. The authors explained this difference as being caused by an increased night-time clearance of cyclosporine, although the AUC after the evening dose was only 10% smaller than the AUC after the morning dose on twice daily dosing (fasting/lack of fasting not reported). An alternative explanation could be dose-dependent pharmacokinetic characteristics of cyclosporine at doses lower than 3 mg/kg (Müller et al., 1994b).

The published studies that aimed to compartmentally characterize cyclosporine pharmacokinetics have described cyclosporine disposition with two (Dunn et al., 2001) or three compartment (Kahan, 1989b) models with initial half-lives ranging from one to two hours and terminal half-lives ranging from five to 27 hours (Dunn et al., 2001; Novartis, 2005). Generally, to achieve therapeutic drug concentrations during the whole dose interval, drugs with a high therapeutic index may be given less frequently than once every half-life. On the other hand, drugs with a narrow therapeutic index should be administered with a dosing interval of one half-life, or more frequently (Rowland and Tozer, 1995). As recommended by the manufacturer (Novartis, 2005), cyclosporine is usually administered in two daily doses. However, in some centers, once-daily or thrice daily dosing are also used.

The major advantage of the once-daily dosing has been better compliance (Schädeli et al., 2002; Tarantino et al., 2004). However, because of the high  $C_{max}$  observed after once-daily administration of cyclosporine, some patients experienced adverse reactions within the first few hours after the drug intake and were consequently switched to a twice-daily regimen (Schädeli et al., 2002). Although not common in adults, thrice daily dosing could be an alternative for those adults with a large clearance and a half-life shorter than 12 hours. Moreover, in young children, who generally have a larger clearance/body weight than do older children (Cooney et al., 1997; del Mar Fernandez De Gatta et al., 2002), the daily dose was divided into three doses in some transplantation centers (Hopppu et al., 1991b; Cooney et al., 1997; del Mar Fernandez De Gatta et al., 2002).

### **Cyclosporine chronopharmacokinetics**

Although some studies have found higher cyclosporine trough concentrations in the morning compared to the evening, these studies have used small numbers of subjects and it is not clear what the cause of the difference is. Changes in liver blood flow, microsomal activity differences, different transporter function, or differences in protein binding during night and day have been suggested as possible causes (Baraldo and Furlanut, 2006). In a study on only five patients, cyclosporine clearance (intravenous administration) was larger in the evening than in the morning (Heifets et al., 1995), and the authors hypothesized that this could be a result of an increase in the activity of the metabolic enzymes in the liver at night. However, Curtis et al. suggested that the metabolic rate stays virtually the same throughout the day, and any differences in cyclosporine concentrations (generally higher morning trough concentrations) are attributable to the fact that patients are more likely to be fasted before the morning dose and fed before the evening dose (Curtis et al., 2006).

### **Cyclosporine drug interactions**

Cyclosporine is a substrate for the drug metabolizing enzymes CYP3A4 and CYP3A5 (Maurer, 1985; Bertault-Peres et al., 1987; Kronbach et al., 1988) and for the efflux drug transporter MDR1 (Goldberg et al., 1988). Moreover, cyclosporine inhibits CYP3A4 and MDR1 both *in vitro* and *in vivo* (Li et al., 1990; Krishna and Mayer, 2000). Cyclosporine has not been directly shown to be a substrate for OATP1B1 and MRP2, but is a relatively potent competitive inhibitor of both these membrane transporters (Chen et al., 1999; Shitara et al., 2003).

Potent inducers of CYP-enzymes and of MDR1 (carbamazepine, phenobarbital, phenytoin, rifampin) all markedly lower cyclosporine blood concentrations and the concurrent use of cyclosporine and these agents should be avoided or accompanied by frequent therapeutic drug monitoring (Langhoff and Madsen, 1983; Freeman et al., 1984; Carstensen et al., 1986; Cooney et al., 1995). Oxcarbazepine treatment with a less pronounced interaction is preferable to carbamazepine and phenobarbital, as oxcarbazepine is only a modest inductor of CYP3A4 (Rösche et al., 2001).

Drugs that increase cyclosporine concentrations are many, and most affect cyclosporine concentrations by inhibiting CYP3A and MDR1. The most important include verapamil (Howard et

al., 1990), diltiazem (Brockmöller et al., 1990), erythromycin (Gupta et al., 1989a), clarithromycin (Ferrari et al., 1994), telithromycin (Sanofi-Aventis, 2007), fluconazole (Ehninger et al., 1989), ketoconazole (el-Agroudy et al., 2004), itraconazole (Kwan et al., 1987), voriconazole (Groll et al., 2004), and the hiv-protease inhibitors (ritonavir, saquinavir, indinavir, lopinavir)(Frassetto et al., 2005).

Generally, cyclosporine increases the concentrations of CYP3A and MDR1 substrates. For example, cyclosporine can increase felodipine (CYP3A4 substrate) concentrations and cause hypotension and swelling of the ankles (Madsen et al., 1996). Cyclosporine also increases the plasma concentrations of all statins, by inhibiting the OATP1B1 in the liver, with possible contributions by CYP3A and MDR1 inhibition (Neuvonen et al., 2006). Furthermore, cyclosporine lowers mycophenolic acid concentrations by inhibiting MRP2 in the liver (Hesselink et al., 2005b).

### **The effects of food and drink on cyclosporine pharmacokinetics**

Grapefruit juice has been shown to increase the bioavailability of cyclosporine, by inhibiting CYP3A in the intestine and causing an increase of up to 40% in the trough concentration (Johnston and Holt, 1995). However, both larger and smaller effect sizes have also been reported (Min et al., 1996). Furthermore, pomelo juice produces similar effects on cyclosporine absorption (Grenier et al., 2006). On the other hand, cranberry or orange juice have no such effect (Grenier et al., 2006).

Food may cause changes in cyclosporine absorption by: affecting cyclosporine transport in the gastrointestinal tract, affecting intestinal motility, or by changes in bile flow. The studies that investigated the effects of food on cyclosporine absorption are discordant. In one study cyclosporine trough concentration,  $C_{max}$  and AUC were increased up to 45% when the oil-based cyclosporine formulation was administered with food compared to when it was administered to fasting patients (Ptachcinski et al., 1985b). However, in another study with the oil-based cyclosporine formulation, food both delayed and impaired the absorption of cyclosporine (Keown et al., 1982). In a third study, the oil-based cyclosporine formulation was administered to: fasting subjects, or to subjects receiving a low-fat meal, or to subjects receiving a high-fat meal. In this study no differences, accountable to fasting or food, were found in the AUC or trough parameters of cyclosporine (Honcharik et al., 1991). A peculiar finding associated with the oil-based formulation of cyclosporine was the appearance of double peaks in the concentration time curve

of the drug. As only 1% of the parent drug is excreted in the bile, entero-hepatic recycling is not a likely explanation for this finding. Rather, the presence of food may cause the second peak, by causing an outflow of bile after a meal and thereby enhancing absorption, especially when given after fasting (Lindholm et al., 1988).

Most published studies on the interactions of cyclosporine and food conducted used the oil-based formulation. The effects of a fat-rich meal on the pharmacokinetics of the oil-based formulation and the microemulsion formulation in 24 healthy volunteers were evaluated in a crossover setting. For the oil-based formulation, a meal containing fatty food nearly doubled the  $T_{max}$  and caused a 37% increase in the AUC compared to the fasting condition. For the microemulsion formulation, fatty food caused no change to the  $T_{max}$ , but the AUC was reduced by 15% compared to administration to fasting subjects (Müller et al., 1994a). In conclusion, the effect of food on the absorption of cyclosporine is equivocal. Due to the possible interactions, cyclosporine should be given either to a fasting patient or consistently with food. Grape fruit juice should not be taken along with cyclosporine.

### **The effects of transplanted organ and disease on cyclosporine pharmacokinetics**

The type of transplanted organ does not appear to have a substantial effect on cyclosporine pharmacokinetics, at least on clearance and volume of distribution values, providing the graft function is good (Dunn et al., 2001). However, in liver transplanted patients, the absorptive part of the small intestine may be diminished (Burckart et al., 1986; Ptachcinski et al., 1986). Bone marrow transplant patients (n=28) with hepatic dysfunction (defined as an elevated [2.0–5.0 mg/dl] bilirubin) had only 60% of the apparent clearance of those patients with normal hepatic function (Yee et al., 1984).

In contrast to the effects of hepatic dysfunction, renal failure does not seem to affect cyclosporine clearance (Dunn et al., 2001). However, the absorption of cyclosporine may be slower in uremic patients than in renal transplant patients with a functioning renal graft (Dunn et al., 2001). The effect of heart disease on cyclosporine pharmacokinetics was evaluated in a small study with only six pediatric patients who had congestive heart failure. In this study, cyclosporine clearance was decreased (0.12 L/h/kg) compared to that of healthy normal children (Burckart et al., 1987). In patients with inflammatory bowel disease, the pharmacokinetic parameters of the cyclosporine

microemulsion formulation were similar to those measured in healthy volunteers (Latteri et al., 2001). Acute gastroenteritis causing diarrhea has been shown to decrease the amount of absorbed cyclosporine to less than half those of normal absorption values (Atkinson et al., 1984).

### **The effects of organ transplantation on cyclosporine pharmacokinetics**

In the uremic patient, gastric motility (Freeman et al., 1985; Kang, 1993), blood protein binding (Reidenberg and Drayer, 1980), and CYP-enzyme and drug transporter function may all be affected (Nolin et al., 2008). After transplantation these processes are eventually normalized, but soon after the operation some perturbations may occur: namely the reduced activity of CYP3A4 and MDR1 in the intestine and liver due to peri-operative inflammation (Haas et al., 2003). In adult patients, the oral clearance of cyclosporine was decreased by 30% within the first months after transplantation (Grevel et al., 1993; Felipe et al., 2003; Jacobson et al., 2003; Wu et al., 2005). In patients who received the oil-based formulation of cyclosporine, the highest mean values for oral bioavailability (about 50%) were found in the first three months after renal transplantation. These declined thereafter to pre-transplantation values within six months (Tufveson et al., 1986; Kahan and Grevel, 1988). The pre-transplantation AUCs of cyclosporine after oral dosing did not correlate well with the corresponding post-transplantation values in adults (Lindholm and Kahan, 1993; Cooney et al., 1994b; Talaulikar et al., 2003). Although the reasons for the change after transplantation in oral bioavailability/apparent clearance are not clear, the authors of the above studies hypothesized that changes after transplantation occur because of changes in gastric emptying rate (Freeman et al., 1985; Kang, 1993), altered bile secretion (Andrews et al., 1985), or alterations in lipoprotein transport in the gastro-intestinal mucosa (Cheema et al., 1987).

### **The effects of development and sex on cyclosporine pharmacokinetics**

The data on cyclosporine pharmacokinetics in children are limited. About 50 publications pertain strictly to cyclosporine related pharmacokinetics, of which only a few have studied the microemulsion formulation. Most of the published studies used small numbers of patients ( $n < 15$ ) and these patients were mostly older than five years of age (age range: 0.5 to 20 years) (Cooney et al., 1997; del Mar Fernandez De Gatta et al., 2002). Furthermore, most of the studies are conducted on oral cyclosporine only and lack long-term follow-up. The main difference in the pharmacokinetics of cyclosporine in children seems to be that the younger patients (age 2–5 years) have about a quarter higher oral daily weight adjusted dose requirement than the older

children (age >10 years). This difference is possibly due to higher clearance per body weight (Cooney et al., 1997; del Mar Fernandez De Gatta et al., 2002). Six published pediatric studies report cyclosporine pharmacokinetic parameters obtained after both intravenous and oral administration of cyclosporine, and the resulting parameters are compiled and given in Table 1.

The mean blood clearance in these patients is 0.58 L/h/kg, which is slightly higher than that generally reported for adults (0.3 to 0.5 L/h/kg) (Ptachcinski et al., 1985a; Ptachcinski et al., 1986; Dunn et al., 2001). The mean volume of distribution is 3.3 L/kg, which is in the same range as reported for adults (3-5 L/kg) (Ptachcinski et al., 1986; Lill et al., 2000). The oral bioavailability of both the oil-based and the microemulsion formulations seem to be the same for adults (Holt et al., 1994) and children (Cooney et al., 1997; del Mar Fernandez De Gatta et al., 2002). In addition, the absorption profile seems to be similar with  $T_{max}$  occurring at about 1-2 hours after the dose in both adults and children for the microemulsion formulation and about at two to four hours after dosing for the oil-based formulation (Ptachcinski et al., 1985a; Ptachcinski et al., 1985b; Awni et al., 1989; Lindholm et al., 1990b; Holt et al., 1994; Friman and Backman, 1996; Cooney et al., 1997; del Mar Fernandez De Gatta et al., 2002).

The role of sex on the disposition of drugs metabolized by CYP3A remains controversial. Although some studies suggest that CYP3A activity in women is higher than in men, evidence to support this hypothesis is discordant (Greenblatt and von Moltke, 2008). No large studies about the sex-related differences in cyclosporine pharmacokinetics have been conducted. However, in one study on 19 stable renal transplant patients (10 women and nine men) no sex-associated differences of cyclosporine pharmacokinetics were seen (Aros et al., 2005).



**Table 1. Published pediatric studies reporting pertinent cyclosporine pharmacokinetic parameters. The inclusion criteria for this table were more than five patients in the study and both intravenous and oral administration of cyclosporine.**

Transplanted Organ	Study period	Age (years)	N	single i.v dose	single p.o. dose	Assay**	CL (L/h/Kg)	Vd (L/Kg)	F (%)	Formulation	References
Kidney	Pre TX*	1.1–16.8	20	3.0 mg/kg/4h	10 mg/kg	RIA	0.52	2.8	21	oil-based	(Hoppu et al., 1991a)
Kidney	Stable post-TX	2–16	7	4.5 mg/kg	13.3 mg/kg	HPLC	0.71	4.7	30.8	oil-based	(Cooney et al., 1994a)
Kidney	Stable post-TX	2–18	13	2.5 mg/kg	2.5 mg/kg	RIA	0.62	2.7	39	oil-based	(Jacqz-Aigrain et al., 1994)
Liver	Immediate post-TX	0.6–5.6	9	2.3 mg/kg	9.6 mg/kg	HPLC	0.56	NA***	8.8	oil-based	(Burckart et al., 1986)
Liver	Stable post-TX	0.5–11	9	10.1 mg/kg/24h	3 x IV dose	RIA	0.72	2.2	37.6	microemulsion	(Dunn et al., 1995)
Liver	Immediate post-TX	0.8–2.5	10	2 mg/kg/24h	5 mg/kg	FPIA	0.37	4.3	22	microemulsion	(Wallemacq et al., 1997)

\*TX = Transplantation

\*\*RIA = radioimmunoassay, HPLC = high pressure liquid chromatography, FPIA = fluorescence polarization immunoassay. The sample matrix for all assays was whole-blood.

\*\*\*NA = Not applicable (parameter not reported)

### Cyclosporine pharmacogenetics

As yet no genetic testing is in use for cyclosporine dose selection prior to transplantation. However, many studies that assessed the associations between cyclosporine pharmacokinetics and genetic polymorphisms have been published (Table 2), but the results remain largely discordant. Studies on the cyclosporine pharmacogenetics in children have not been published.

**Table 2. Previously published positive associations between DNA sequence variations and cyclosporine pharmacokinetics in adults.**

N	PK* data	Time after TX**	Studied subjects	SNP	Effect on CL/F†	References
75	AUC <sub>0-4</sub>	First month	Renal TX patients	<i>ABCB1</i> c.3435T	CL/F ↑	(Foote et al., 2006)
19	AUC <sub>12h</sub>	>1 month	Renal TX patients	<i>ABCB1</i> c.3435T	CL/F ↑	(Yates et al., 2003)
88	C0, C2	First month	Renal TX patients	<i>ABCB1</i> c.3435T	CL/F ↓	(Azarpira et al., 2006)
44	C2	First month	Liver TX patients	<i>ABCB1</i> c.3435T	CL/F ↓	(Bonhomme-Faivre et al., 2004)
106	AUC <sub>0-4</sub>	>1 month	Renal TX patients	<i>ABCB1</i> c.1236T	CL/F ↑	(Anglicheau et al., 2004)
151	AUC <sub>0-4</sub>	>1 month	Kidney and heart TX patients	<i>CYP3A4</i> g.-392G	CL/F ↑	(Hesselink et al., 2004)
14	AUC <sub>24h</sub>	NA***	Healthy volunteers	<i>CYP3A4</i> g.-392G	CL/F ↑	(Min and Ellingrod, 2003)
50	C0	>1 month	Renal TX patients	<i>CYP3A5</i> g.6986A	CL/F ↑	(Haufrond et al., 2004)
106	C0	>1 month	Renal TX patients	<i>CYP3A5</i> g.6986A	CL/F ↑	(Hu et al., 2006)

\*PK = Pharmacokinetic

\*\*TX = Transplantation

\*\*\*NA = Not applicable

†CL/F = Presumed apparent clearance based on C0, C2, or AUC values.

Although patients that expressed the CYP3A5 enzyme have a higher apparent tacrolimus clearance, such a finding is not clear for cyclosporine (Haufrond et al., 2004). In one study, healthy volunteers who expressed CYP3A5 had a lower cyclosporine AUC than did those patients that did not express CYP3A5 (Min et al., 2004). In another study, renal transplant recipients that expressed CYP3A5 had lower dose-corrected cyclosporine trough levels than did those patients that did not express CYP3A5 (Haufrond et al., 2004). However, these findings could not be confirmed in other studies on renal and heart transplant patients (Hesselink et al., 2003; Anglicheau et al., 2004; Hesselink et al., 2004; Zhao et al., 2005).

With regard to *CYP3A4* polymorphisms, Hesselink et al. found that renal and heart transplant recipients who carried the *CYP3A4* g.-392G allele had 9% higher apparent cyclosporine clearance than did non-carriers (Hesselink et al., 2004). Another study on healthy volunteers obtained a similar finding (Min and Ellingrod, 2003). However, as with *CYP3A5*, the findings of Hesselink and Min and Ellingrod could not be replicated in other studies (Rivory et al., 2000; von Ahsen et al., 2001; Hesselink et al., 2003).

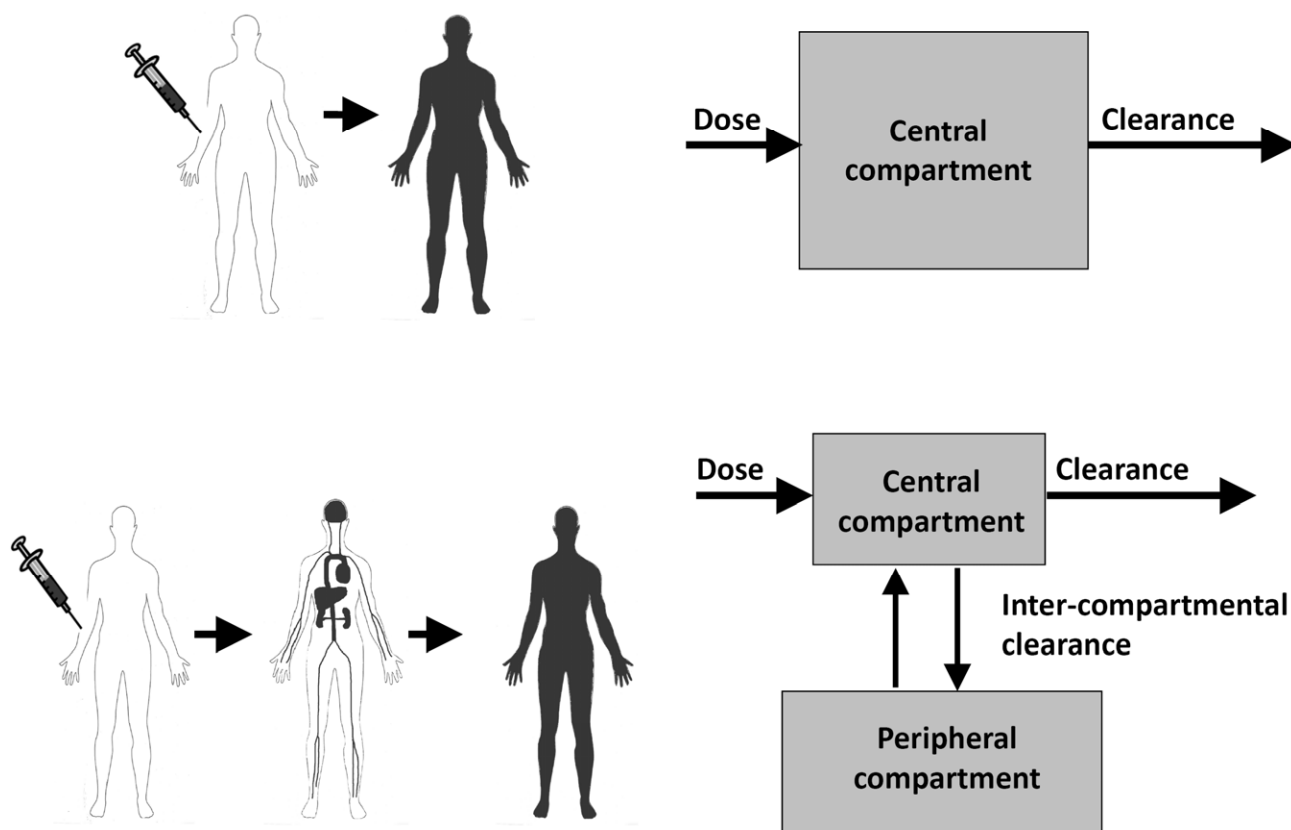
The results regarding the effects of *ABCB1* polymorphisms on cyclosporine pharmacokinetics are also discordant. In a study on 106 adult renal transplant recipients, the patients who carried the *ABCB1* c.1236C>T variant allele had a 15% larger AUC<sub>0-4</sub> than did non-carriers (Anglicheau et al., 2004). In 44 liver transplant recipients, the carriers of the *ABCB1* c.3435T allele had higher dose-corrected C2 levels and needed a 50% lower cyclosporine dose than did non-carriers (Bonhomme-Faivre et al., 2004). In contrast, for 19 renal transplant recipients who carried the *ABCB1* c.3435T allele, the cyclosporine apparent clearance was higher than for non-carriers (Yates et al., 2003). Several other studies have found no associations between cyclosporine pharmacokinetics and *ABCB1* genotypes (Hesselink et al., 2005a; Chinn and Kroetz, 2007). As the synonymous c.3435C>T variant has no effect on protein sequence, the c.3435T allele has been shown to have functional consequences only in those haplotypes that include the c.1236T or c.2677T allele, or both (Kimchi-Sarfaty et al., 2007).

Haplotype analysis, rather than investigating a single SNP genotype, is an approach that has been suggested to clarify the role of *ABCB1* polymorphisms. However, the published results about the effects of *ABCB1* haplotypes on cyclosporine pharmacokinetics have not been able to clarify the role of *ABCB1* polymorphisms. In a study on six heart transplant patients, the carriers of the *ABCB1* c.1236T-c.2677T-c.3435T haplotype had a 30% larger AUC<sub>0-12</sub> than did non-carriers (Chowbay et al., 2003). In a study on 112 renal transplant patients, the carriers of the c.1236T-c.2677T-c.3435C haplotype had higher dose-adjusted trough blood concentrations than did non-carriers (Wang et al., 2009). In a third study on 103 renal transplant recipients, the dose-adjusted trough concentrations of cyclosporine were higher in carriers of the *ABCB1* haplotype c.1236C-c.2677A-c.3435C than in non-carriers (Qiu et al., 2008).

## 6. PHARMACOKINETIC MODELING AND POPULATION PHARMACOKINETICS

In order to depict drug concentrations as a function of time, simple mathematical models may be used, although the real course of the drug in the body is a complex process (Speight and Holford, 1997). In pharmacokinetic modeling, the body is generally depicted as a system of compartments, even though these lack physiological or anatomical reality (Rowland and Tozer, 1995). In the one-compartment model, the body is depicted as one homogenous unit. The two-compartment open model consists of a central compartment (which includes the blood or plasma) and a peripheral compartment (which includes the tissues). The three-compartment open model consists of a central compartment and two peripheral compartments.

**Figure 5. A schematic description of the one- and two-compartment pharmacokinetic models.**



In the case of the one-compartment pharmacokinetic model (upper part of the figure), drug distribution is considered to occur instantaneously. In the two-compartment model the administered drug distributes quickly to the central compartment and more slowly to the peripheral compartment.

Non-compartmental pharmacokinetic analysis is a simple approach to calculate summary statistics for a pharmacokinetic study. However, it can be difficult to apply when there are many dosing occasions and when the concentration data are sparse, as is the case of therapeutic drug monitoring. Using individual compartmental modeling for analyzing therapeutic drug monitoring (TDM) data does not make the situation easier because of the sparseness of the data and because some individuals may be consistent with one type of model, but others with a different type of model. For example, most patients may be more consistent with a 2-compartment model, but others with a 3-compartment model.

In the early 1980s, Lewis Sheiner and Stuart Beal described a new approach to pharmacokinetic data analyses, which was later called 'population pharmacokinetics' (Sheiner and Beal, 1980; Sheiner and Beal, 1981; Sheiner and Beal, 1983). Population pharmacokinetics is a statistical technique that involves the simultaneous estimation of population mean and variance parameters of merged data obtained from all studied individuals (Davidian and Giltinan, 1995; Ette and Williams, 2004a). This approach makes it possible for such information to be shared among individuals. It also enables parameter estimates to be obtained even for those individuals for whom there are too few observations to allow estimation by alternative standard methods. Thus it enables the use of sparse data from each individual, but for reliable estimates a large number of individuals must be included (Davidian and Giltinan, 1995; Ette and Williams, 2004a).

The statistical technique used in population pharmacokinetics is called nonlinear mixed effects modeling. The word 'mixed' refers to fixed and random effects. The fixed effects are the population average values of pharmacokinetic parameters and the random effects quantify the extent of pharmacokinetic variability that is not explained by the fixed effects (Whiting et al., 1986). Conceptually, the population pharmacokinetic model can be viewed as comprising three sub-models: structural, stochastic and covariate (Whiting et al., 1986). The structural sub-model describes the overall time-course of the concentrations (e.g. by a one or a two-compartment model). The stochastic sub-model describes the variability between patients (inter-individual variability), the variability between occasions (inter-occasion/within-patient/intra-individual variability), and the residual variability. The covariate sub-model expresses relationships between covariates and model parameters. For example, it describes how total clearance increases with increasing weight (Ette and Williams, 2004a).

## REVIEW OF LITERATURE

Since the introduction of population pharmacokinetics, its use increased exponentially in the 1990s after the development of powerful microprocessors (Bonate, 2005). In publications describing cyclosporine pharmacokinetics, the population pharmacokinetic approach has gained further ground in the 2000s and has mostly been used in adult studies (Leger et al., 2002; Hesselink et al., 2004; Bourgoin et al., 2005; Wu et al., 2005). Nonetheless, only two pediatric population pharmacokinetic studies on cyclosporine have currently been published (Saint-Marcoux et al., 2006; Irtan et al., 2007).

## AIMS OF THE STUDY

In order to optimize cyclosporine dosing and monitoring in renal transplanted children, and to anticipate the need for individual dose modifications, it is important to understand how developmental, clinical, and genetic factors contribute to the variability of cyclosporine pharmacokinetics. At the Hospital for Children and Adolescents in Helsinki Finland, pharmacokinetic profiles and therapeutic drug monitoring data have been collected for almost 20 years from nearly 200 renal transplanted children. These data provide an exceptional opportunity to comprehensively assess the factors that affect cyclosporine pharmacokinetics in renal transplanted children. Maximum information content can be obtained from those data by using robust analytical methods for this task, such as population pharmacokinetics.

The specific aims of the study were to:

- 1) characterize the magnitude of the effects of developmental and clinical factors on cyclosporine clearance, volume of distribution and oral bioavailability both before and after renal transplantation,
- 2) evaluate whether DNA sequence variations in genes that (may) influence major pharmacokinetic pathways of cyclosporine (*ABCB1*, *ABCC2*, *SLCO1B1*, *CYP3A4*, *CYP3A5*, or *NR1I2*) are associated with the pharmacokinetics of cyclosporine in pediatric renal transplanted patients, and whether the effects of these variants are related to age and
- 3) explore the possibilities for cyclosporine dosing optimization in children.

## PATIENTS AND METHODS

### 1. DATA COLLECTION

#### Study population

The whole study population consisted of 176 renal transplant patients (116 boys and 60 girls). All but two of the patients were Caucasians and Finnish. All patients had end-stage renal disease: 67 patients had congenital nephrotic syndrome of the Finnish type (NPHS1), 21 patients had urethral valve disease, 14 patients had nephronophthisis, 11 patients had polycystic renal disease, and the remaining 63 patients had diagnoses that were more rare than the above mentioned diseases. The number of patients and the basic patient characteristics in each of the studies are presented in Table 3. More detailed patient characteristics are presented in Table 4 (before transplantation,  $n=175$ ) and in Table 5 (after transplantation,  $n=137$ ). For modeling purposes, the whole dataset containing both the pre- and post-transplantation data was divided into an estimation dataset ( $n=162$ ) and a semi-external validation dataset ( $n=93$ ), i.e. the validation dataset included 14 new patients that were not included in the estimation dataset, and 79 patients who had sufficient data to be divided between the estimation and validation datasets.

In study I ( $n=162$ ), the pre-transplantation data of the patients were studied to obtain a population pharmacokinetic model including covariate effects to explain the variability in cyclosporine pharmacokinetic parameters. This model was used to calculate the individual clearance, oral bioavailability and volume of distribution estimates. These were subsequently used to assess associations with genetic polymorphisms in a subset of the children studied prior to renal transplantation (study II,  $n=104$ ). In study III ( $n=176$ ), post-transplantation follow-up pharmacokinetic data of the same patients as in study I and II were added to the previous datasets and a model describing both the pre-transplantation and the post-transplantation cyclosporine pharmacokinetics was developed. Finally, the pharmacokinetic model developed in study III was used to assess the post-transplantation associations between cyclosporine pharmacokinetics and genetic polymorphisms.



**Table 3. Number of patients and basic patient characteristics in studies I, II, and III**

Variable	Study I	Study II	Study III
Total number of patients	162	104	176
Oral pre-transplantation pharmacokinetic data (n)	89	60	89
Intravenous pre-transplantation pharmacokinetic data (n)	162	102	172
Oral post-transplantation pharmacokinetic data (n)	NA*	NA	137
Intravenous post-transplantation pharmacokinetic data (n)	NA	NA	84
Mean $\pm$ SD body weight (kg)	22.2 $\pm$ 15.7	18.3 $\pm$ 12.1	28.4 $\pm$ 16.7
Mean $\pm$ SD Age (years)	6.3 $\pm$ 5.7	4.8 $\pm$ 4.6	8.2 $\pm$ 5.5
Mean $\pm$ SD Hematocrit (%)	31 $\pm$ 7	33 $\pm$ 6	32 $\pm$ 5
Mean $\pm$ SD Serum creatinine ( $\mu$ mol/L)	592 $\pm$ 252	528 $\pm$ 231	149 $\pm$ 184
Mean $\pm$ SD Plasma cholesterol (mmol/L)	5.7 $\pm$ 1.6	5.6 $\pm$ 1.5	5.5 $\pm$ 1.2

In study I the population pharmacokinetics of cyclosporine was studied in patients awaiting renal transplantation.

In study II the pharmacogenetics of cyclosporine was studied in patients awaiting renal transplantation.

In study III the population pharmacokinetics of cyclosporine were studied in the combined dataset of patients that had data both before and after renal transplantation. In addition, in study III the pharmacogenetics of cyclosporine were studied after renal transplantation.

\*NA = Not applicable (data not collected)

**Table 4. Characteristics of 175 patients at the time of the pre-transplantation study ( $1.1 \pm 0.9$  years before renal transplantation).**

Variable	Estimation dataset (n=162)			Validation dataset (n=13)		
	n	Mean $\pm$ SD	Range	n	Mean $\pm$ SD	Range
Body weight (kg)	162	22.2 $\pm$ 15.7	6.9 – 64	13	19.2 $\pm$ 13.4	8.3 – 42.1
Age (years)	162	6.3 $\pm$ 5.7	0.36 – 17.5	13	5.0 $\pm$ 4.8	0.8 – 12.9
Hematocrit (%)	148	31 $\pm$ 7	15 – 48	13	34 $\pm$ 8	16 – 44
Serum creatinine ( $\mu$ mol/L)	145	592 $\pm$ 252	165 – 1669	13	453 $\pm$ 115	170 – 600
Plasma cholesterol (mmol/L)	132	5.7 $\pm$ 1.6	3.1 – 9.8	13	4.5 $\pm$ 0.9	2.5 – 6.4
Height (cm)	145	107.3 $\pm$ 35.5	61.0 – 176	NA*	NA	NA
Blood hemoglobin (g/L)	148	104 $\pm$ 23	47 – 154	NA	NA	NA
Serum protein content (g/L)	126	64 $\pm$ 8	42 – 83	NA	NA	NA
Serum prealbumin (mg/L)	119	373 $\pm$ 92	179 – 668	NA	NA	NA
Serum aspartate aminotransferase (U/L)	128	35 $\pm$ 23	10 – 247	NA	NA	NA
Serum alanine aminotransferase (U/L)	129	34 $\pm$ 54	5 – 564	NA	NA	NA
Serum alkaline phosphatase (U/L)	132	682 $\pm$ 407	104 – 1952	NA	NA	NA
Total plasma triglycerides (mmol/L)	133	2.9 $\pm$ 1.5	0.68 – 8.2	NA	NA	NA
Low density lipoprotein cholesterol (mmol/L)	112	3.3 $\pm$ 1.3	1.2 – 7.4	NA	NA	NA
High density lipoprotein cholesterol (mmol/L)	128	1.1 $\pm$ 0.3	0.47 – 2.3	NA	NA	NA

Of the 176 children in the whole study population 175 had pre-transplantation data.

The pre-transplantation data of the patients in study III were exactly the same as in study I.

Of the 14 new patients included in the validation dataset, 13 had pre-transplantation data.

\*NA = Not applicable (data not collected)

**Table 5: Body weight, age, hematocrit, serum creatinine and plasma cholesterol values in 137\* pediatric patients during the post-transplantation follow-up.**

Variable	Group	Estimation dataset (n=132)**			Validation dataset (n=93)		
		n	Mean $\pm$ SD	Range	n	Mean $\pm$ SD	Range
<b>Body weight (kg)</b>	First month after transplantation	82	23.9 $\pm$ 15.8	8.8 – 71.4	2	39.1 $\pm$ 6.0	31.5 – 47.0
	1-3 months after transplantation	28	29.9 $\pm$ 19.8	9.3 – 77.3	33	29.2 $\pm$ 17.6	8.9 – 72.8
	3-12 months after transplantation	34	34.0 $\pm$ 21.5	9.4 – 90.4	48	25.4 $\pm$ 17.1	8.5 – 76.2
	more than 1 year after transplantation	106	37.7 $\pm$ 16.4	9.8 – 91.5	69	34.0 $\pm$ 14.9	10.9 – 76.2
<b>Age (years)</b>	First month after transplantation	82	6.3 $\pm$ 5.4	0.7 – 18.2	2	11.9 $\pm$ 1.6	10.3 – 13.5
	1-3 months after transplantation	28	7.4 $\pm$ 5.7	1.3 – 18.4	33	8.5 $\pm$ 5.9	0.8 – 18.4
	3-12 months after transplantation	34	8.9 $\pm$ 5.9	1.4 – 18.6	48	7.1 $\pm$ 5.0	1.0 – 18.1
	more than 1 year after transplantation	106	11.6 $\pm$ 4.6	2.0 – 20.2	69	10.2 $\pm$ 4.2	1.9 – 19.6
<b>Hematocrit (%)</b>	First month after transplantation	82	28 $\pm$ 4	15 – 41	2	29 $\pm$ 4	23 – 35
	1-3 months after transplantation	28	34 $\pm$ 5	26 – 57	33	34 $\pm$ 5	23 – 44
	3-12 months after transplantation	34	35 $\pm$ 5	17 – 49	48	35 $\pm$ 6	18 – 53
	more than 1 year after transplantation	106	35 $\pm$ 4	21 – 46	69	34 $\pm$ 5	23 – 56
<b>Serum creatinine (<math>\mu</math>mol/L)</b>	First month after transplantation	82	262 $\pm$ 271	11 – 1144	2	191 $\pm$ 126	63 – 500
	1-3 months after transplantation	28	63 $\pm$ 27	18 – 137	33	83 $\pm$ 31	39 – 165
	3-12 months after transplantation	34	72 $\pm$ 28	21 – 159	48	79 $\pm$ 36	38 – 301
	more than 1 year after transplantation	106	107 $\pm$ 67	23 – 798	69	102 $\pm$ 34	35 – 274
<b>Plasma cholesterol (mmol/L)</b>	First month after transplantation	82	5.7 $\pm$ 1.2	3.5 – 9.0	2	4.1 $\pm$ 0.8	3.2 – 4.8
	1-3 months after transplantation	28	5.6 $\pm$ 1.1	3.8 – 7.6	33	5.8 $\pm$ 1.3	3.2 – 10.0
	3-12 months after transplantation	34	5.5 $\pm$ 1.0	3.8 – 7.6	48	5.7 $\pm$ 1.4	3.2 – 12.7
	more than 1 year after transplantation	106	4.9 $\pm$ 1.0	2.6 – 8.1	69	5.1 $\pm$ 0.9	3.1 – 7.4

\*Of the 176 patients in the whole population, 137 provided post-transplantation follow-up pharmacokinetic data with the microemulsion cyclosporine formulation

\*\*Of the 137 patients with post-transplantation follow-up data, 132 were included in the estimation dataset, and the remaining five patients were among the "new patients" included in the validation dataset

### **Ethical considerations**

The study protocol was approved by the Ethics Committee for Pediatrics, Adolescent Medicine and Psychiatry of the Hospital District of Helsinki and Uusimaa. For genetic testing, a written informed consent was obtained from: the patient's parents, both child and parents, or from the child only (age>16 years).

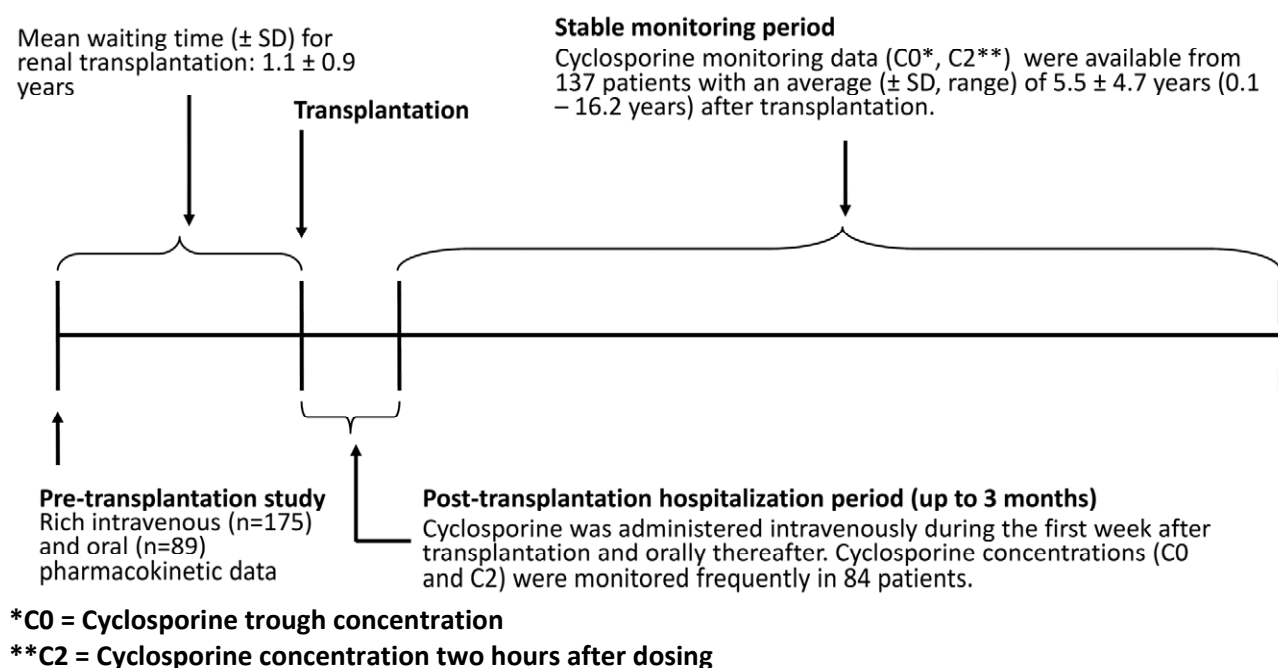
### **Pharmacokinetic and covariate data collection**

At the Hospital for Children and Adolescents of the University of Helsinki, cyclosporine medication was started pre-operatively with an individual pharmacokinetically determined dose that was aimed at achieving a trough cyclosporine concentration of 300 µg/L immediately after renal transplantation. Since 1988 the individual starting doses of cyclosporine in children have been determined using predictions obtained by pre-transplantation pharmacokinetic studies (Hopppu et al., 1991b). In the pharmacokinetic study, the patients entered the clinic in the morning after an overnight fast. Cyclosporine was administered both intravenously and orally. Cyclosporine was given as an intravenous 4-h infusion (3 mg/kg, Sandimmune, Novartis, Basel, Switzerland) and as either a single oral dose [10 mg/kg, Sandimmune (oil-based oral formulation) or Sandimmune Neoral (microemulsion liquid formulation), Novartis] with an interval of at least 24 hours between the doses (usually 48-72 hours). Cyclosporine concentrations were determined from one ml EDTA blood samples drawn at 0, 1, 2, 3, 4, 6, 9, 12, 16 and 24 hours after the oral dose, and before (0 h), in the middle of (2 h) and at the end of the intravenous infusion (4 h) and then at 1, 2, 3, 4, 6, 9, 12, 16 and 24 h after the end of infusion. The exact time for each blood sample was documented. Samples of blood were drawn through an indwelling cannula, held open by an obturator or by slow glucose infusion. The blood samples were stored at +4°C and analyzed within three days.

The pre-transplantation pharmacokinetic studies were conducted 1.1±0.9 years before transplantation, during the 1988–2006 period. Intravenous data from 162 children and oral data from 89 of the same patients receiving the microemulsion formulation (used since 1995) were included in study I. The data obtained from the patients who had received the oil-based oral formulation of cyclosporine, were excluded. In study III, intravenous data were available from 175 children and data for orally administered cyclosporine from the same 89 children as in study I.

Post-transplantation pharmacokinetic data obtained during the 1995–2006 period were collected from 137 children (study III). Intravenous post-transplantation pharmacokinetic data were available the first few days after renal transplantation. The switch to oral dosing was usually carried out during the first post-operative week. The oral post-transplantation pharmacokinetic data for the microemulsion formulation were collected. These data included both the liquid (used mainly in young children) and the capsule formulation. Follow-up therapeutic drug monitoring data after renal transplantation consisted of 3189 trough concentrations and also 1044 concentrations after the dose, mostly two hours post dose. The exact times of the post-dose concentrations ranged from 0.73 to 5.25 hours after the dose and gave a mean of  $2.3 \pm 0.4$  hours. In addition, 69 dose interval studies from 67 patients, performed one to three years after transplantation, were included. On such occasions, blood was drawn before and 1, 2, 3, 4, 6, and 8 hours after the morning dose of cyclosporine for t.i.d. dosing and, in addition, 12 hours post-dose for b.i.d. dosing regimens. The patients provided follow-up data for 3.0 years (median), with a range from 0.1 to 10.3 years after transplantation (25<sup>th</sup> and 75<sup>th</sup> percentiles 0.79 and 6.0 years). The follow-up lasted for up to 4.0 years (median), with a range from 0.1 to 16.2 years after transplantation (25<sup>th</sup> and 75<sup>th</sup> percentiles 1.5 and 9.6 years). Cyclosporine doses, concentrations, exact times of cyclosporine concentrations, and clinical covariate data were collected from patient records. An illustration of the data collection period is presented in Figure 6.

**Figure 6. Schematic description of the data collection period.**



## Clinical treatment protocol

Before transplantation, children with congenital nephrotic syndrome of the Finnish type (NPHS1) were nephrectomized bilaterally. All children were on continuous ambulatory or cycling peritoneal dialysis for at least three months before transplantation. The cyclosporine target trough concentration was 300  $\mu\text{g/L}$  immediately after renal transplantation. The target trough was gradually reduced to 100  $\mu\text{g/L}$  after six months. C2 concentrations of 1500–1800  $\mu\text{g/L}$  early after transplantation, and 800–1000  $\mu\text{g/L}$  six months after transplantation were considered as additional targets. Cyclosporine was administered as t.i.d. doses in children younger than eight years and as b.i.d. doses in older children. Additional immunosuppressive medication consisted of azathioprine or mycophenolate mofetil (after 1999) and methylprednisolone.

## Cyclosporine assay

During the first month after transplantation, whole blood cyclosporine concentrations were analyzed using the fluorescence polarization immunoassay (FPIA) technique (TDX, Abbot, Illinois, USA) due to its availability at weekends. Cyclosporine blood concentrations before transplantation and after the hospitalization period were determined by a specific monoclonal radioimmunoassay (RIA) technique [Sandimmune Kit, Sandoz, Basel, Switzerland until May 1994; Cyclo-Trac Kit, Incstar, Stillwater MN, USA until December 1999 and Cyclo-Trac SP-Whole Blood, DiaSorin,

Saluggia, Italy] thereafter. The Cyclo-Trac kits provided by Incstar and DiaSorin are technically the same kit. The limits of detection of these assays were approximately 5 µg/L, and the within-run and between run coefficients of variation were <5% for FPIA and <7% for RIA, for concentrations above 30 µg/L. The laboratory participates in the Cyclosporine International Proficiency Testing Scheme, for quality control. The FPIA has more residual cyclosporine metabolite interference than does the specific radioimmunoassay. The degree to how much the FPIA measured values differ from those values measured by the RIA method is dependent on the individual metabolite profile and the concentration sampling time. The values measured by FPIA gives from 0% to about 20% greater cyclosporine concentration values than those obtained by RIA (Morris et al., 1992; Aspeslet et al., 1997; Rodriguez et al., 2005).

## 2. PHARMACOKINETIC MODELING

### General description of population pharmacokinetic modeling and model estimation

Pharmacokinetic modeling was conducted using nonlinear mixed effects models (Davidian and Giltinan, 1995; Ette and Williams, 2004b) exemplified here by the simple case of a one-compartment, first-order elimination model for single bolus dose intravenous administration:

$$\text{Concentration}(\text{time}) = \text{Dose}/V_d \times e^{-CL/V_d \times \text{time}} + \epsilon,$$

where the parameter vector that describes the pharmacokinetic properties of the drug in question is composed of CL (clearance) and V<sub>d</sub> (volume of distribution), the dose and the time are the input variables and  $\epsilon$  (epsilon) describes the additive residual error between the model predicted concentrations and the observed concentrations. More generally, a function describing the  $j^{\text{th}}$  concentration observation in individual  $i$  ( $y_{ij}$ ) can be written:

$$y_{ij} = f(x_{ij}, \theta) + \epsilon_{ij},$$

where  $f(\dots)$  is a function that describes the transfer from input  $x$  ( $x_{ij}$  is the  $j^{\text{th}}$  input for the  $i^{\text{th}}$  individual) to observations  $y$ .  $\theta$  (theta) denote the model parameters, and  $\epsilon_{ij}$  is the residual error (unexplained variability) for the  $j^{\text{th}}$  observation of the  $i^{\text{th}}$  individual. The values of  $\epsilon_{ij}$  are assumed to

## PATIENTS AND METHODS

be normally distributed with a mean of zero and a variance of  $\sigma^2$  (sigma). To account for inter-individual and inter-occasion (intra-individual) variability, components associated to the  $\theta$  parameter vector can be estimated.

Model estimation was performed by using the nonlinear mixed effects modeling computer program, NONMEM (version 5 in studies I and II and version 6 in study III, GloboMax LLC, Hanover, MD, USA). The computer program PsN (Lindbom et al., 2005) was used for implementing computer intensive statistical methods in NONMEM. The computer program Xpose 3.1 (in study I and II) and Xpose 4.0 (in study III) (Jonsson and Karlsson, 1999) were used for graphical diagnostics. The comparison of competing models was done using the objective function value (OFV) in the likelihood ratio test. The analyses were performed with the first-order conditional estimation method with interaction. For those runs, the inclusion criterion for a statistically significant covariate was a  $\geq 10$ -point drop in the OFV (corresponding to  $P \leq 0.001$ , loss of one degree of freedom). The genetic covariate screening runs (study III) were carried out using the first-order method to reduce the length of run times. For these runs, a randomization test (Manly, 1997) was performed to elucidate a decline in OFV corresponding to a  $P=0.05$ , with loss of two degrees of freedom. To obtain symmetric errors, the concentration data were log-transformed for the pharmacokinetic analyses.

### **Modeling of the pre-transplantation pharmacokinetic data (study I)**

The structural pharmacokinetic model was initially established, followed thereafter by development of the covariate model. The stochastic (random variability) model was refined interactively during the whole model development. Defining the structural model involved evaluation of the 1-, 2-, and 3-compartment models. The compartmental model was parameterized in terms of clearances and volumes with rate constants used to describe the absorption process. Both first order and zero order absorption models, with and without lag-time, were assessed. In addition, an alternative absorption model capable of describing a delayed absorption, the transit-compartment model (Savic et al., 2007), was tested. Models including a description of the saturable cyclosporine blood binding with a Michaelis-Menten type model were also evaluated.



On the basis of the intravenous data obtained, possible covariate effects were evaluated for body weight (BW), height, body surface area (calculated by the method described by Gehan-George) (Gehan and George, 1970). Other parameters analyzed were: age, diagnosis, blood hemoglobin, hematocrit, serum creatinine, serum protein content, serum prealbumin, serum alanine aminotransferase (log-transformed due to skewed distribution), serum alkaline phosphatase, total plasma triglycerides, total plasma cholesterol, low density lipoprotein and high density lipoprotein (Table 4). Missing covariate values were assigned the population median value.

The non-continuous covariates that showed a correlation with a pharmacokinetic parameter and all continuous parameters were entered into the model and evaluated by the NONMEM program. The non-continuous covariates were modeled by assessing the change in the typical value for each category, exemplified here by the typical value of clearance (TVCL) and diagnosis (DIAG). The diagnosis covariate was coded as follows:

NPHS1 (DIAG=1), urethral valve (DIAG=2), polycystic renal disease (DIAG=3), nephronophtisis (DIAG=4), other diagnoses (DIAG=5).

The effect of the diagnosis covariate on typical value of clearance was coded as follows:

$$TVCL = \theta_1 \times (1 + IND),$$

where the  $\theta_1$  represents the estimate of clearance in a typical individual with NPHS1. If DIAG=1, then IND=0, if DIAG=2, then IND= $\theta_2$ , if DIAG=3, then IND= $\theta_3$ , if DIAG=4, then IND= $\theta_4$ , if DIAG=5, then IND= $\theta_5$ . The theta values represent the fractional change in clearance for each diagnosis.

The continuous covariates were modeled as a linear function, and parameterized so that the relationship was centered on the median covariate value, exemplified here by the typical value of clearance (TVCL) and total plasma cholesterol (CHOL):

$$TVCL = \theta_6 \times [1 + \theta_7 \times (CHOL - CHOL_{\text{median value}})]$$

In this case,  $\theta_6$  represents the estimate of clearance in a typical individual with median plasma cholesterol, and  $\theta_7$  the fractional change in clearance with each unit change in plasma cholesterol.

The covariate-parameter relationships were evaluated one at a time, using a common covariate effect, *i.e.* the same  $\theta_7$ , for all volume and clearance parameters. For statistically significant covariate-parameter relationships ( $\geq 10$  point drop in the OFV), a model allowing different covariate coefficients for clearance and volume parameters was evaluated.

The stochastic model comprised an evaluation of the residual error, inter-individual variability (IIV), and inter-occasion variability (IOV). The residual error was initially described using a slope-intercept model. The residual error model was also assessed for the impact of the assay method and also IIV for the magnitude of the residual error. An exponential model was used to describe the IIV of the pharmacokinetic parameters and covariance between individual parameters was assessed.

### **Modeling of the combined pre- and post-transplantation pharmacokinetic data (study III)**

For study III a dataset combining pharmacokinetic and covariate data both before and after transplantation, was collated. Thereafter, the dataset containing both the pre- and post-transplantation data was divided into an estimation dataset ( $n=162$ ) and a semi-external validation dataset ( $n=93$ ). The validation dataset included 14 new patients. There were pre-transplantation data for 13 of these patients. The fourteenth patient had only post-transplantation data. In addition to these new patients, the validation dataset had data obtained from 79 patients which were sufficient to be divided between the estimation and validation datasets. Data from these 79 patients contributed to the estimation dataset with their pre-transplantation data and data collected after 2001, when C2 monitoring was initiated in our clinic. The rest of their data were included in the validation dataset.

Model building that was initiated was based on the previous pre-transplantation pharmacokinetic model. The post-transplantation data were first fitted with the pre-transplantation model. The resulting structural model was then challenged and the stochastic parts of the model were re-evaluated. Finally the covariate model was updated. Defining the optimal structural model involved evaluation of one-, two-, and three-compartment models, whereas the existing covariate effects (body weight, serum creatinine, serum cholesterol, and hematocrit on clearance and volume of distribution) were retained in the model. In the random variability model building

process, inter-individual variability, inter-occasion variability, and residual error were evaluated. The IIV model was re-assessed by inclusion of random variability on all the structural model parameters (allowing covariance between IIV parameters) and the residual error parameters. The IIV model was reduced in steps on the basis of objective function value and actual estimates of random parameters. IOV on oral bioavailability and clearance was assessed and modeled by an additional random effect on IIV as described by Karlsson and Sheiner (Karlsson and Sheiner, 1993). The effect of age on IOV was further evaluated. Components of the previous residual error model were challenged and additional components referring to the analytical methods were assessed.

Missing covariate values for serum and cholesterol and hematocrit were handled by carrying the previous observations for the patient forward in the post-transplantation time period. As in study I, body weight was included as a covariate for clearance and volume parameters in the model using allometric scaling (Holford, 1996). The effects of hematocrit, plasma cholesterol and serum creatinine on clearance and volume of distribution of cyclosporine were also modeled as in study I, with a linear function parameterized so that the relationship was centered on the median covariate value. The rest of the covariate modeling was focused on modeling oral bioavailability and how time after transplantation, age and dosing frequency affected this parameter.

Initially, the effect of time after transplantation (PTXT) on oral bioavailability (F) was modeled by assessing the F value at different stages: before transplantation, in the first month after transplantation, and in the post-transplantation time thereafter. Instead of discrete values for the time periods, a continuous change in F with PTXT was implemented in the final model with an equation analogous to that of the simple one compartment first order absorption model. In addition to PTXT, patient age (modeled linearly) was observed to affect F: F increased with increasing age. However, when the dosing frequency of cyclosporine (modeled categorically) was added to the model, the age covariate was no longer statistically significant. Furthermore, to test the effect of dosing frequency on oral bioavailability, the subpopulation of children that provided pharmacokinetic data both for twice daily and thrice daily dosing was studied separately. A similar effect size, attributable to the dosing frequency, was found in the subpopulation as in the whole population.

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The effect of dosing frequency on oral bioavailability was challenged and modeled instead using a Michaelis-Menten type dose-dependent, saturable oral bioavailability model either with or without the PTXT covariate effect. However, the categorically modeled dosing frequency of cyclosporine and PTXT combined as assessed by the reduction in OFV (<-100) described the data much more accurately than the dose-dependent oral bioavailability model with or without the PTXT covariate effect. In the final model, the oral bioavailability was modeled as follows:

$$F(\text{PTXT}, \text{DF}) = F_{\text{TV}} + (\theta_8 / (\theta_8 - \theta_9)) \times (e^{-\theta_9 \times \text{PTXT}} - e^{-\theta_8 \times \text{PTXT}}) \times \theta_{10}^{\text{DF}},$$

where  $F_{\text{TV}}$  is the typical population value of oral bioavailability before transplantation, PTXT is the time after transplantation in years, and  $\theta_8$  and  $\theta_9$  define the steepness of the ascent and descent of the resulting curve, respectively.  $\theta_{10}$  is the dosing frequency covariate effect and DF is either 0 or 1 for patients on twice and thrice daily dosing, respectively. To limit the value of oral bioavailability between 0 and 1, the whole oral bioavailability function was logit-transformed:

$$F = e^{F(\text{PTXT}, \text{DF})} / (1 + e^{F(\text{PTXT}, \text{DF})}).$$

### Empirical Bayes estimate based analyses

According to the Bayesian statistical approach, population estimates should include prior knowledge into an estimate of a true population value. Hence, Bayes estimates attempt to make a compromise between what was observed and what is theorized. For population pharmacokinetic models, individual parameter estimates are regularly obtained as empirical Bayes (posthoc) estimates (EBE) (Bonate, 2006). The EBE estimates are individual estimates of the model parameters calculated, which are based on the population model and the concentration data of the individual patient. These estimates are used in model diagnostics, covariate screening, and for illustrative purposes in plots. However, EBEs of the inter-individual random effect,  $\eta$  (eta), are biased (shrunk) toward the population mean, when the individual data for the parameter are not rich (frequent concentration data). This shrinkage, called  $\eta$ -shrinkage, can be estimated as  $1 - (\text{SD}_{\text{EBE}}/\omega)$ , where  $\text{SD}_{\text{EBE}}$  is the standard deviation of the EBEs and  $\omega$  (omega) is the population model estimate of the standard deviation of  $\eta$ . When the value of  $\eta$ -shrinkage increases from zero toward one, the parameter EBE approaches the population mean, and the value of EBE as a diagnostic variable decreases (Karlsson and Savic, 2007).

In study I, EBEs of clearance, volume of distribution and oral bioavailability in different age groups were studied and the effect of adjusted clearance by body weight, allometrically scaled body weight or body surface area were also studied. In addition to the normalization methods used in study I, a new analysis using the pre-transplantation EBE values of clearance was conducted to demonstrate the effect of normalization of clearance by liver size. Clearance was adjusted by the estimates of liver volume, obtained by the empirically derived meta-analysis based formula (Liver volume =  $0.722 \times \text{BSA}^{1.176}$ ) (Johnson et al., 2005). In study II, the population pharmacokinetic model developed in study I was used to estimate the individual EBEs of the primary pharmacokinetic parameters of cyclosporine (clearance, oral bioavailability, volume of distribution) in those patients for which genetic data were available. Allometrically scaled clearance values (divided by body weight<sup>3/4</sup>) were used in all statistical analyses. Allometric scaling has been successfully applied to the pharmacokinetic modeling of substrates that undergo liver metabolism in patient populations that combine data obtained from children and adults (Anderson et al., 2000; Capparelli et al., 2003; Rajagopalan and Gastonguay, 2003; Björkman, 2006). According to allometric principles for estimating the metabolic rate of the body, i.e. clearance, the following equation is used  $\text{CL} = \text{CL}_{\text{standard}} \times (\text{individual weight/standard weight})^{3/4}$ . For estimating the volume of distribution the following equation is used  $\text{Vd} = \text{Vd}_{\text{standard}} \times (\text{Individual weight/standard weight})^1$  (West et al., 1997).

The hepatic and pre-hepatic extraction ratios of cyclosporine were calculated using the following equations:

$$\text{Hepatic extraction ratio} = \text{Systemic blood clearance EBE} / \text{Hepatic blood flow (Q}_H\text{)}$$

$$\text{Pre-hepatic extraction ratio} = 1 - \text{Oral bioavailability EBE} / (1 - \text{Hepatic extraction ratio})$$

In children,  $\text{Q}_H$  correlates well with body surface area (Murry et al., 1995). As the allometric scaling method produces values similar to those estimated by using body surface area, we used the following equation to scale adult  $\text{Q}_H$  (1.5 l/min/70 kg) to children:

$$\text{Q}_{H,\text{Child}} = \text{Q}_{H,\text{Adult}} \times (\text{Body weight}_{\text{Child}} / 70 \text{ kg})^{3/4}$$

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In study III, the pre-transplantation EBE of clearance and oral bioavailability were compared with the corresponding post-transplantation values both immediately and long after renal transplantation in order to assess how well the pre-transplantation pharmacokinetics of cyclosporine predict post-transplantation pharmacokinetics. Correlations were studied with linear regression and prediction errors were calculated as follows:

$$\text{Prediction error} = (\text{Pre-TX EBE} - \text{Post-TX EBE}) / \text{Post-TX EBE}$$

### **Model validation, calculation of standard errors, and the randomization test**

The model validity was evaluated at every step of the model building process by using goodness-of-fit-plots as a basic tool (Karlsson and Savic, 2007). Population pharmacokinetic goodness-of-fit-plots involve plotting the model predicted concentrations against the true observed concentrations. Another basic diagnostic tool is to plot the weighted residuals against time. These standard model diagnostics are helpful in identifying possible model misspecification. In addition, standard model validation involved checking the parameter estimates after every run for unrealistic values. In study III, the final population model was also validated by conducting a visual predictive check (Karlsson and Holford, 2008) with both the estimation dataset and the semi-external validation dataset. The visual predictive check is a simulation based diagnostic tool, used to determine whether a model can reproduce the variability in the observed data.

The standard errors for the final population model parameters in studies I and III were obtained by a bootstrap method (Efron and Tibshirani, 1993). This is a general method for measuring statistical accuracy and precision. The bootstrap involves creating new datasets by sampling from the original dataset. The new datasets are then used for the model estimation similarly to the original data. Depending on the statistics of interest, different numbers of resampled datasets are needed. Estimation of the relative standard errors typically require about 100 bootstrap datasets. In studies (I and III) the bootstrap resampling for the final population model was repeated 100 times, and values of the parameters were used to calculate relative standard errors for the final model parameter estimates. The standard errors obtained within NONMEM were used for the genetic covariate test runs (study III).

Randomization tests for the runs judged to be significant by the likelihood ratio test were conducted, to determine the  $\Delta\text{OFV}$  that corresponds to a preset  $P$ -value for the genetic runs performed by the FO-estimation method. If there was no true relationship between a covariate and a parameter, it would not matter what covariate value was assigned to an individual. In the randomization test the individual covariate vectors were randomly permuted, while maintaining a relevant structure of the pharmacokinetic and covariate data. A full model, including a parameter–covariate relationship, was then fitted to those data with randomly permuted covariate values, and the  $\Delta\text{OFV}$  from the fit of the reduced model was determined. This was done by iteration ( $n=1000$ ), with an automated script in PsN, to obtain an empirical reference distribution for the  $\Delta\text{OFV}$  under the null hypothesis (Manly, 1997; Lindbom et al., 2005).

### **Medication potentially interacting with cyclosporine**

In the pre-transplantation study (intravenous and oral cyclosporine dosing) eight patients received medications that potentially interacted with cyclosporine. One patient received carbamazepine during the study, two patients received oxcarbazepine, and five patients received phenobarbital. In order to assess the effect of these eight patients on the population parameters, we deleted them from the population dataset after the final pre-transplantation model was estimated. Thereafter, the population parameters were estimated again, and virtually no changes were observed. Thus, these patients' data were left in the final dataset. In the post-transplantation phase five patients received phenobarbital, two received carbamazepine, four received oxcarbazepine, and one patient received roxithromycin medication on at least one study occasion. Because the number of patients with medications that potentially interacted with cyclosporine was small, we could not assess the medications as a covariate on clearance or oral bioavailability. The patients with interacting medication were subsequently deleted from the population dataset after the final model was estimated, to assess the effect these patients' data had on the population parameters. The population parameters were then re-estimated and only small changes were observed. Thus, the data from these patients were left in the final dataset. In the pharmacogenetic dataset (study II) four patients, each with only intravenous data, who received medication that potentially interacted with cyclosporine (carbamazepine [ $n=1$ ], oxcarbazepine [ $n=1$ ] and phenobarbital [ $n=2$ ]), were excluded from statistical analyses.

### **3. PHARMACOGENETIC ANALYSES**

#### **Patients included in the pharmacogenetic analyses**

In total 104 patients who had previously undergone the pre-transplantation pharmacokinetic study were also recruited into the genetic testing for those polymorphisms that possibly affect cyclosporine pharmacokinetics. These patients were genotyped for 17 sequence variations in six candidate genes (Table 6), that putatively could affect cyclosporine pharmacokinetics. Intravenous pre-transplantation pharmacokinetic data were available for 102 of the 104 children. Both intravenous and oral pre-transplantation pharmacokinetic data obtained on the microemulsion formulation were available for 58 patients. In study III, all data from the estimation and validation dataset for 91 patients who had both genetic and post-transplantation pharmacokinetic data were included for post-transplantation analyses.

#### **Description of the genotyping**

Blood samples of between 2 to 4 ml volumes were drawn from 104 inpatients and genomic DNA was extracted by standard methods (QIAamp DNA Blood Mini Kit, Qiagen, Hilden, Germany). The genotyping for all variants was carried out by allelic discrimination with TaqMan 5'-nuclease assays (Applied Biosystems, Foster City, CA). Genotypes were verified by re-genotyping 16 randomly selected samples (15% of the patients), which resulted in a 100% concordance with the original genotype.



**Table 6.** The genetic sequence variations studied for all the 104 patients in study II. For each variation the location, data base SNP ID number, amino acid changes (when applicable), and sequences of primers and probes or Applied Biosystems assay identification numbers are presented.

Gene	Location	Variation	dbSNP ID*	Amino acid change	Primers and probes or AB assay ID number
ABCB1	Exon 12	c.1199G>A	rs2229109	Ser400Asn	AB assay ID: C__15951365_20
	Exon 13	c.1236C>T	rs1128503	Synonymous	AB assay ID: C__7586662_10
	Exon 22	c.2677G>T/A	rs2032582	Ala893Ser/Thr	5'-GACAAGCACTGAAAGATAAGAAAGAACT-3' 5'-GTAGGGAGTAACAAAATAACACTGATTAGAAAT-3' 5'-VIC-CCTTCCCAGCACCT-MGB-3' 5'-FAM-CTTCCCAGAACCTT-MGB-3' 5'-NED-CTTCCCAGTACCTTC-MGB-3'
ABCB1	Exon 26	c.3435C>T	rs1045642	Synonymous	5'-ATTGCTGAGAACATTGCCTATGG-3' 5'-CAGTGACTCGATGAAGGCATGTAT-3' 5'-VIC-CTCACGATCTCTT-MGB-3' 5'-FAM-CCTCACAAATCTCTT-MGB-3'
					5'-TTGATGAAACAAGTAAAGAAAGAAACACAA-3'
					5'-CAGAACTTCTCCAGCATGATTCCT-3'
ABCC2	Exon 10	c.1446C>G	**	Synonymous	5'-VIC-CTGGAACGAAAGACTC-MGB-3' 5'-FAM-CTGGAACAAAGACTC-MGB-3' 5'-GTGATGGTGCTTGTAATCCCAATT-3' 5'-CTGGGTGACTTTTCTTTACCTGAATG-3'
					5'-VIC-ATACTGTCCACCAAGAGT-MGB-3'
					5'-FAM-CTGTCCACGAAGAGT-MGB-3'
SLCO1B1	Exon 5	c.521T>C	rs4149056	Val174Ala	5'-GAAACACTCTTATCTACATAGGTGTTTA-3' 5'-CCCCTATTCCACGAAGCAT-3' 5'-VIC-TACCCATGAACACATATA-MGB-3' 5'-FAM-TACCCATGAACGCATATA-MGB-3'

Gene	Location	Variation	dbSNP ID*	Amino acid change	Primers and probes or AB assay ID number
CYP3A4	Exon 1, promoter	g.-392A>G	rs2740574		5'-TGGAATGAGGACAGCCATAGAGA-3'
					5'-AGTGGAGCCATTGGCATAAATCT-3'
					5'-VIC-AAGGGCAAGAGAGAG-MGB-3'
					5'-FAM-AAGGGCAGGAGAG-MGB-3'
CYP3A4	Exon 7	c.566T>C	rs4987161	Phe189Ser	AB assay ID: C__27859822_10
CYP3A4	Exon 7	c.666T>C	rs55785340	Ser222Pro	5'-GTGGAAAACACCAAGAAGCTTTTAAAGA-3'
					5'-GGTGATTATATCTCAATAAAGCAGTTATTTTAAAGAGAG-3'
					5'-VIC-ACATACTTATTGAGAGAAAG-MGB-3'
					5'-FAM-CATACTTATTGGGAGAAAG-MGB-3'
CYP3A5	Intron 3	g.6986A>G	rs776746		5'-ATGGAGAGTGGCATAGGAGATACC-3'
					5'-GGTAATGTGTCCAAACAGGG-3'
					5'-VIC-TGTCCTTCAGTATCTTT-MGB-3'
					5'-FAM-TGTCCTTCAATATCTCTT-MGB-3'
NR1I2	Exon 1, 5' UTR	g.-25385C>T	rs3814055		AB assay ID: C__27504984_30
NR1I2	Exon 1, 5' UTR	g.-24381A>C	rs1523127		AB assay ID: C__263841_20
NR1I2	Intron 1	g.-205_-200del GAGAAG	rs3842689		5'-GGTCCTGCACTAGCCTCCTA-3'
					5'-GTGACTTGTGGTAACTCATGCAGTA-3'
					5'-VIC-CAGGAGAAGCCCTTAAC-MGB-3'
					5'-FAM-CACCACAGCCTTAAC-MGB-3'
NR1I2	Intron 5	g.7635A>G	rs6785049		AB assay ID: C__29280426_10
NR1I2	Exon 6, promoter	g.8055C>T	rs2276707		AB assay ID: C__15882324_10

\*The Single Nucleotide Polymorphism database identification number

\*\*SNP described in (Niemi et al., 2006)

### Statistical analysis of the pharmacogenetic data in study II

Departure of genotype frequencies from the Hardy-Weinberg equilibrium was tested for as described by Guo and Thompson (Guo and Thompson, 1992) using the software ARLEQUIN version 2.000 (available from URL:<http://anthro.unige.ch/software/arlequin/>). *ABCB1*, *CYP3A*, and *NR1I2* haplotypes were inferred using an algorithm based on Bayesian inference with the software PHASE version 2.1.1 (Stephens et al., 2001; Stephens and Donnelly, 2003). All genotype frequencies were in equilibrium with the Hardy-Weinberg distribution, except for the *ABCB1* c.1236C>T SNP (excess of heterozygotes,  $P = 0.030$ ). In total eight haplotypes were inferred from the *ABCB1* SNPs, five haplotypes from the *CYP3A* SNPs, and seven haplotypes from the *NR1I2* SNPs.

Comparisons of the pharmacokinetic variables of cyclosporine between subjects with different genotypes or haplotypes were made using one-way analysis of variance (ANOVA). The EBE values of cyclosporine clearance, oral bioavailability, and pre-hepatic and hepatic extraction ratios were normally distributed (Shapiro-Wilk test). The non-parametric Kruskal-Wallis test was applied for the statistical comparison of the volume of distribution, because this variable is not normally distributed. In order to investigate whether the effects of sequence variations depend on age, we performed a sub-group association analysis after dividing the population into three age groups (< 1 year, 1 – 8 years, and > 8 years), for SNPs and haplotypes with a minor allele frequency of > 30%. The sample size of 98 was estimated to be sufficient to detect a 15%/30% change in clearance for heterozygous/homozygous patients with a statistical power of over 80% at an alpha level of 5%. At this level the minor allele frequency was at least 7.2% assuming a Hardy-Weinberg distribution of genotypes. To detect a 15%/30% change in oral bioavailability for heterozygous/homozygous patients, the statistical power was estimated to be over 80% with a sample size of 58, when the minor allele frequency was at least 34%. Possible relationships between oral bioavailability, hepatic extraction ratio, and pre-hepatic extraction ratio were investigated using Pearson's correlation coefficient.  $P < 0.05$  was considered statistically significant. No correction for multiple testing was used. These statistical analyses were done using SPSS for Windows, version 13.0 (Chicago, Illinois).

## Statistical analysis of the pharmacogenetic data in study III

In study III, SNPs and haplotypes in the *ABCB1*, *SLCO1B1*, *ABCC2*, *CYP3A4*, *CYP3A5* and *NR1I2* genes that had a variant allele frequency over 7% were included in the genetic analyses. We used the final population model that described cyclosporine pharmacokinetics in children before and after transplantation to model the effects of the genetic polymorphisms on the pharmacokinetic parameters as a linear function of the number of alleles. For instance, a change in clearance in *ABCB1* c.1236T allele carriers compared to the non-carriers was evaluated using the following equation:

$$\begin{aligned} \text{Typical value of clearance} = & \text{Typical value of clearance before transplantation} \times (1 + \theta_{11})^{\text{IND}} \\ & \times (1 + \theta_{12} \times \text{GEN})^{\text{IND}}, \end{aligned}$$

where IND is 0 and 1 for pre- and post-transplantation, respectively, and GEN is the genetic polymorphism in question, coded as GEN=0 (non-carrier), GEN=1 (heterozygous carrier), and GEN=2 (homozygous carrier).  $\theta_{11}$  is the fractional difference in clearance between the post-transplant non-carrier patients and the pre-transplant value, and  $\theta_{12}$  is the fractional change in clearance for each variant allele after transplantation. Oral bioavailability was similarly modeled, though also taking into account the additional effects of time after transplantation and dosing frequency after transplantation. This parameterization in the model was considered necessary, as the post-transplantation non-carrier group values may have differed from the typical value of clearance/bioavailability in the pre-transplantation group. The effects of the genetic polymorphisms on the pharmacokinetics of cyclosporine were studied in the whole genetic dataset population (n=91). The effects of genetic polymorphisms were also separately studied in data from patients before eight years of age (n=58) and after eight years of age (n=65), to discover possible age-related changes.

## RESULTS

### 1. THE POPULATION PHARMACOKINETIC MODEL

We used population pharmacokinetic modeling to study the effects of developmental, clinical, and genetic factors on cyclosporine pharmacokinetics in a total of 176 subjects (age range: 0.36–20.2 years) before and up to 16 years after renal transplantation, to improve cyclosporine dose individualization in children. The modeling resulted in a linear three-compartment model with first order absorption without a lag-time. This model adequately described the structural model (Figure 7) for the pre- and post-transplantation pharmacokinetic data. The population parameter values for the pre- and the combined pre- and post-transplantation pharmacokinetic models are presented in Table 7.

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**Table 7:** Parameter estimates with relative standard errors\* (RSE) describing cyclosporine pharmacokinetics in 162 patients before transplantation and the combined pre- and post transplantation pharmacokinetics of cyclosporine (n=176).

Parameter	Pre- transplantation		Pre- and Post-transplantation	
	Estimate	RSE (%)	Estimate	RSE (%)
<b>Typical values</b>				
Clearance <sup>†</sup> (L/h)	6.1	1.4	5.3	2.5
Central compartment volume <sup>†</sup> (L)	5.3	4.6	5.1	4.9
1 <sup>st</sup> intercompartmental clearance <sup>†</sup> (L/h)	1.5	2.8	1.4	3.9
1 <sup>st</sup> peripheral compartment volume <sup>†</sup> (L)	19.6	4.6	21.4	10.0
2 <sup>nd</sup> intercompartmental clearance <sup>†</sup> (L/h)	3.0	6.2	2.5	5.8
2 <sup>nd</sup> peripheral compartment volume <sup>†</sup> (L)	4.4	3.7	4.1	4.5
Oral bioavailability <sup>‡</sup> (F)	0.36	4.7	0.385	4.0
Absorption rate constant (Ka) (1/h)	0.68	5.0	NA	NA
<b>Covariate effects</b>				
Cholesterol covariate effect (L/mmol)	-0.054	23.2	-0.0333	29.2
Creatinine covariate effect (L/μmol)	0.00021	26.3	0.000272	19.1
Hematocrit covariate effect (1/%)	-0.0073	34.1	-0.00726	25.3
Time after transplantation covariate effect, $\theta_1$ on F	NA**	NA	39.5	18.4
Time after transplantation covariate effect, $\theta_2$ on F	NA	NA	2.92	23.3
Dosing frequency covariate effect, $\theta_3$ on F	NA	NA	0.653	10.9
<b>Inter-individual variability (IIV) parameters</b>				
IIV clearance (CV)***	0.17	2.3	0.14	12.9
IIV 1 <sup>st</sup> peripheral compartment volume (CV)	0.42	12.8	0.46	20.4
IIV 1 <sup>st</sup> intercompartmental clearance (CV)	0.31	4.5	0.25	14.7
IIV absorption rate constant (CV)	0.33	8.0	0.42	21.3
IIV Oral bioavailability (CV)	0.31	47.0	0.21	24.7
<b>Inter-occasion variability in oral bioavailability (CV)</b>				
	NA	NA	0.18	9.8
<b>Residual error parameters</b>				
Intravenous proportional error (CV)	0.089	5.7	0.10	7.0
Intravenous additive error (SD in μg/L)	1.5	22.0	2.1	15.5
IIV intravenous residual error (CV)	0.43	9.1	0.39	29.4
Oral proportional error (CV)	0.2	7.4	0.27	2.7
IIV oral residual error (CV)	0.53	17.1	0.23	25.3
Analytical method covariate effect for the RIA <sup>††</sup> Sandimmune kit	2.6	11.3	2.3	12.0
Analytical method covariate effect for the FPIA <sup>‡‡</sup> method	NA	NA	4.5	11.0

\*Relative standard error = standard error of parameter estimate / parameter estimate × 100%. \*\*NA, Not applicable

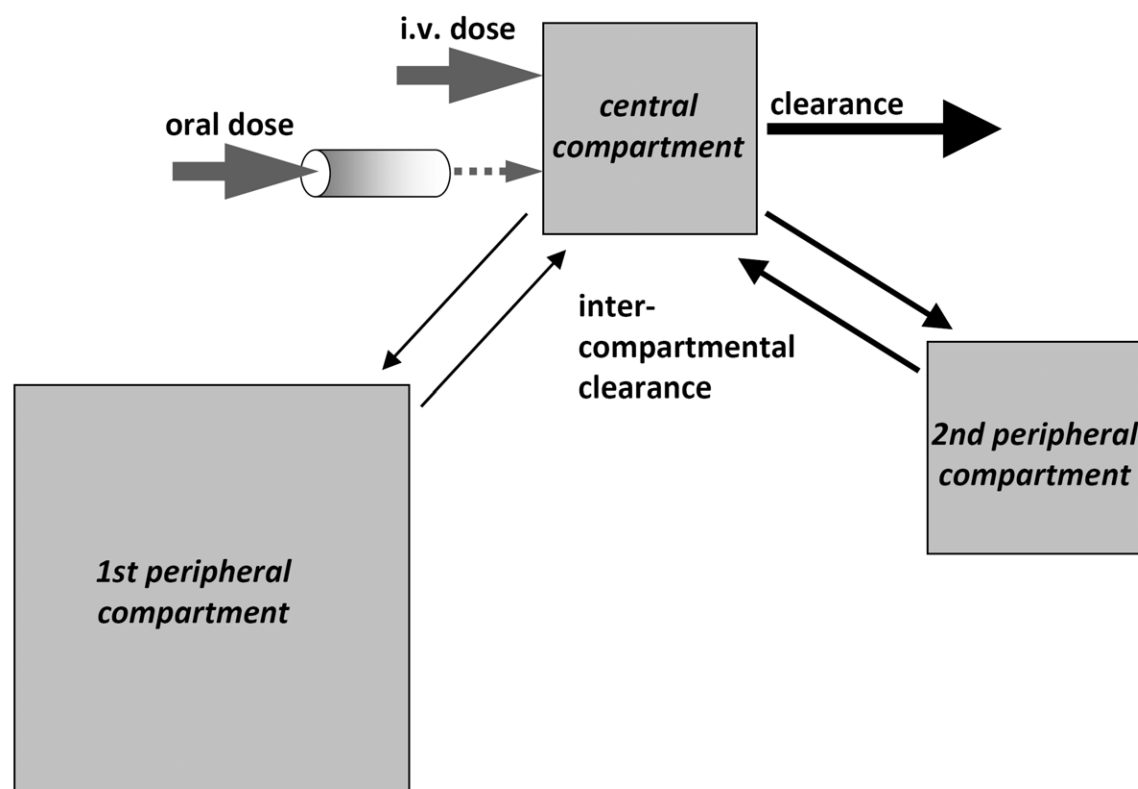
\*\*\*CV, Coefficient of variation = standard deviation / mean

<sup>†</sup>The typical values refer to a patient with median covariate values (e.g. body weight of 13 kg) in the pre-transplantation situation/at the time of transplantation. The data in the whole population could be described according to the following covariate model: Typical value = Typical parameter estimate × (Body weight/13)<sup>A</sup> × [1 - 0.0333 × (Cholesterol - 5.1)] × [1 - 0.00726 × (Hematocrit - 34)] × [1 + 0.000272 × (Serum creatinine - 97)]; A = 3/4 for clearance parameters and A = 1 for volume parameters.

<sup>‡</sup> The typical value refers to patients in the pre-transplantation situation. After transplantation, the oral bioavailability was modeled with the time after transplantation and the dosing frequency covariates. Oral bioavailability = Typical value of pre-transplantation oral bioavailability + ( $\theta_1 / (\theta_1 - \theta_2)$ ) × (e<sup>-0.2 × PTXT</sup> - e<sup>-0.1 × PTXT</sup>) ×  $\theta_3^{DF}$ , where DF is 0 and 1 for patients on twice and thrice daily dosing, respectively. The whole oral bioavailability function was logit-transformed.

<sup>††</sup> Specific radioimmunoassay (RIA) and <sup>‡‡</sup> fluorescence polarization immune assay (FPIA) for quantification of cyclosporine concentrations. The residual error magnitude for the RIA Sandimmune kit was 2.3 times and the FPIA kit was 4.5 times larger, respectively, than that for the newer RIA kit by DiaSorin.

**Figure 7. Schematic description of the three compartment pharmacokinetic model used to describe the population pharmacokinetics of cyclosporine.**



The sizes of the boxes are relative to the compartmental volumes, and the thickness of the arrows are relative to the clearances depicted in Table 7.

#### **The pre-transplantation model covariates and the stochastic model (study I)**

Cyclosporine clearance and volume of distribution were related to body weight by using an allometric model, with fixed values for the exponents ( $3/4$  for clearance and 1 for volume of distribution) obtained from the literature (Holford, 1996). The other statistically significant covariates ( $\Delta\text{OFV} \geq 10$ ) that affected clearance and volume of distribution were hematocrit, plasma cholesterol and serum creatinine. Clearance and volume parameters increased, with increasing body weight and serum creatinine. Clearance and volume parameters decreased, with increasing plasma cholesterol and hematocrit. Allometrically scaled body weight alone accounted for 4-fold differences in cyclosporine clearance and 6-fold differences in volume parameters. In addition, serum creatinine, hematocrit and total plasma cholesterol were estimated to explain up

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to 20%–30% of inter-individual differences in clearance and volume of distribution. No significant covariates for oral bioavailability were discovered.

Inter-individual variability (IIV) was assigned to all pharmacokinetic parameters. However, three parameters (the central and second peripheral compartment volumes and the corresponding intercompartmental clearance) shared the same parameter for IIV, but allowing for different magnitudes of IIV. The residual error for intravenous administration was described by a proportional and an additive component in combination whereas for oral administration it was described solely by a proportional component. The addition of an IIV component to the residual error further improved the model. Nevertheless, the addition of inter-occasion variability to the model parameters did not improve the model. Moreover, the influence of using an older and also a newer specific radioimmunoassay for quantification of cyclosporine concentrations was best taken into account by introducing this factor as a covariate for residual error. In order to account for model under-prediction, at the 15 to 24 hours post-dose time points after oral administration, the final model for oral administration was assigned a 23% smaller typical value of clearance than that for intravenous administration.

### **The combined pre- and post-transplantation pharmacokinetic model (study III)**

A linear three-compartment model with first-order absorption without lag time was used as the structural model to describe the data for the pre-transplantation pharmacokinetic model. Because we only had a few concentration measurements in the early absorption phase ( $t=0-1\text{h}$ ) in the post-transplantation time period, the absorption rate constant was fixed to the value obtained from the pre-transplantation study. Only the inter-individual variability for this parameter was estimated. A reduced IIV model was sufficient for the combined pre- and post-transplantation model compared to the pre-transplantation model. Thus, IIV was assigned: to total clearance, to one of the inter-compartmental clearances, to one of the compartmental volumes, to the absorption rate constant, and to the oral bioavailability. The effects of using the FPIA cyclosporine assay method in the first month after transplantation and two different RIA assays at other times were modeled by including the different assay methods as covariates into the residual error. A systematic covariate effect of using FPIA versus RIA could not be assessed, as simultaneous measurements for both assay methods were available from 13 patients only. Inter-occasion



variability was assigned to oral bioavailability and was found to be about as large as the IIV for this parameter (CV  $\approx$  20%). Inter-occasion variability did not change with age.

### **Model validation**

In every step of the model building process standard goodness-of-fit plots and parameter value assessments were conducted to assure optimum model validity. The data of the pre- and post-transplantation studies were combined for the final model (study III), and model evaluation was also carried out by a visual predictive check. The visual predictive check ascertained that the average model prediction matched the observed concentrations and that the model predicted variability was reasonable both before and after transplantation for both the estimation and validation datasets.

### **The model covariates in study III**

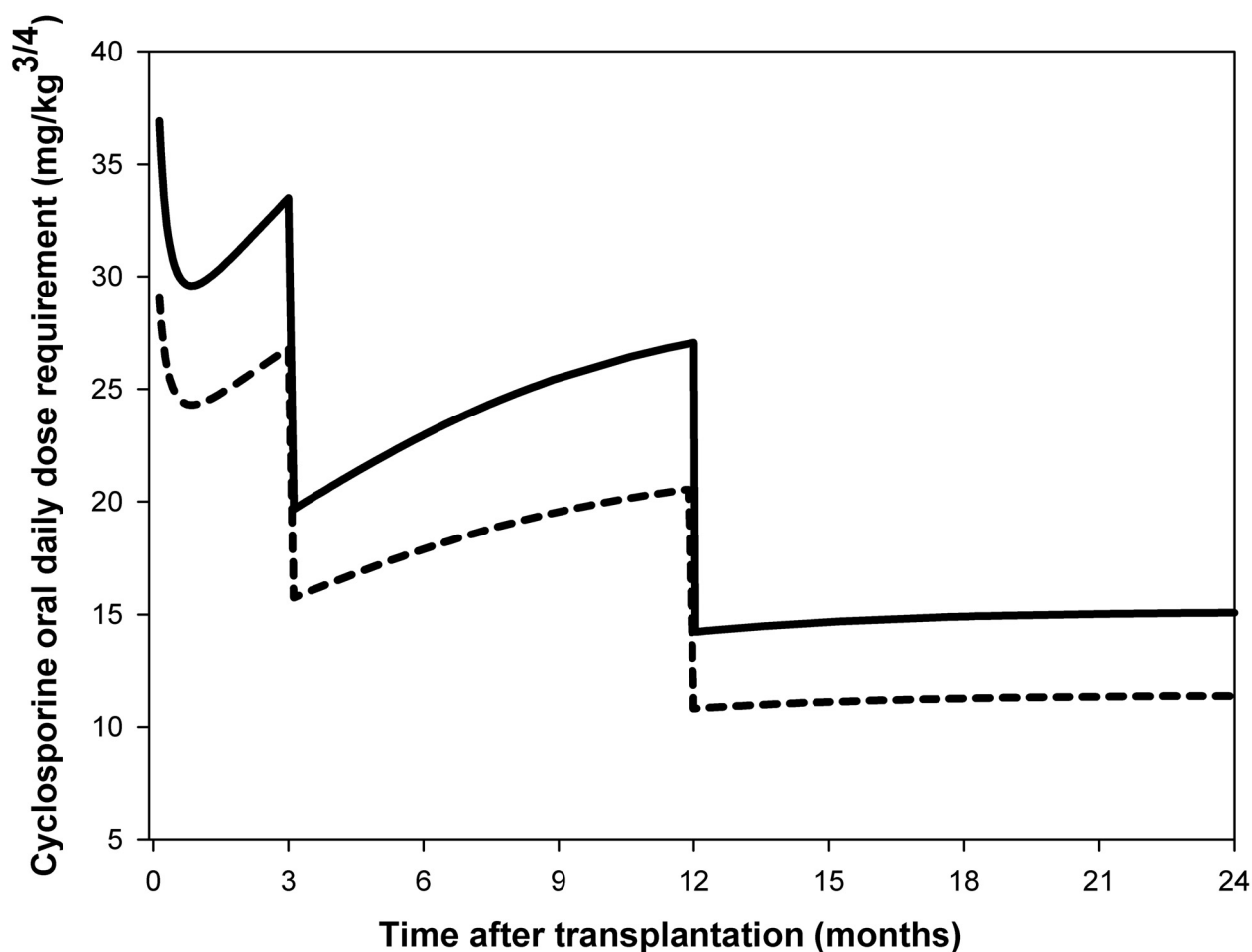
Body weight (modeled allometrically) was the most significant covariate for absolute clearance and volume of distribution. The covariate effects of serum creatinine, hematocrit and plasma cholesterol were re-estimated and found to be statistically significant (OFV reduced by  $>35$  for each covariate) in a similar manner to that found for the pre-transplantation model. However, in the post-transplantation situation their effect was only small as the variabilities in the covariates are also much smaller after transplantation than before. Thus, when plasma cholesterol decreased by one mmol/L, hematocrit decreased by 4% units, and serum creatinine increased by 100  $\mu$ mol/L: whereas the typical values of clearance and volume of distribution increased by 3% only.

In the post-transplantation situation, the time after transplantation (modeled nonlinearly using two parameters) and dosing frequency (b.i.d. or t.i.d. dosing, modeled categorically) were assigned as covariates to the bioavailability of cyclosporine (OFV fell by  $>150$ , for both covariates, i.e.  $P<0.0001$ ). The bioavailability increased rapidly after renal transplantation, reaching the maximal values of 59% and 48% at about one month in patients on b.i.d. and t.i.d. dosing, respectively. Thereafter, bioavailability decreased gradually to reach the pre-transplantation values of 39% and 29% approximately 1–1.5 years after transplantation in b.i.d. and t.i.d. dosing, respectively. Patients on b.i.d. dosing consistently had a 1.25–1.3 times higher bioavailability than did patients on t.i.d. dosing. The clinical significance of body weight, time after transplantation and dosing frequency is shown in Figure 8. Moreover, the model based starting doses of cyclosporine

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for a typical dose administration scheme (i.v. dosing over the first three days, followed by a switch to oral thrice daily dosing on day four), are presented in Table 8.

**Figure 8: Typical, model based allometrically scaled oral daily dose ( $\text{mg/kg}^{3/4}$ ) requirement† of cyclosporine for children in the first two years after transplantation, calculated on the basis of target trough concentrations.**



Dashed line, twice daily dosing (based mainly on data from older children on capsule formulation). Solid line, thrice daily dosing (based mainly on data from young children on liquid formulation).

†The oral daily dose requirement was calculated on the basis of using the model based clearance (CL) and oral bioavailability (F) in the equation: Daily dose requirement =  $(\text{CL}/\text{F}) \times \text{AUC}_{\text{req}}$ , where  $\text{AUC}_{\text{req}}$  is the mean daily area under the concentration-time curve (AUC) of cyclosporine required to reach the typical target trough concentrations in the study population. The  $\text{AUC}_{\text{req}}$  was 23000  $\mu\text{g}\cdot\text{h}/\text{L}$  during the first three months after transplantation, corresponding to a target trough of 350  $\mu\text{g}/\text{L}$ , 13400  $\mu\text{g}\cdot\text{h}/\text{L}$  three months to one year post-transplantation, corresponding to a target trough of 200  $\mu\text{g}/\text{L}$ , and 7000  $\mu\text{g}\cdot\text{h}/\text{L}$  after the first year post-transplantation, corresponding to a target trough of 100  $\mu\text{g}/\text{L}$ .

**Table 8. Model based typical cyclosporine single dose requirement\* in the first week after renal transplantation for children on thrice daily dosing to reach a target trough concentration of 300 – 350 µg/L.**

Body weight	1 <sup>st</sup> post-operative day, intravenous dosing, single dose (mg/kg)	4th post-operative day, oral dosing, single dose (mg/kg)
10	3.3 – 3.8	5.9 – 6.9
15	3.0 – 3.5	5.4 – 6.3
20	2.8 – 3.2	5.0 – 5.8
25	2.6 – 3.1	4.7 – 5.5
30	2.5 – 2.9	4.5 – 5.3
35	2.4 – 2.8	4.3 – 5.1
40	2.3 – 2.7	4.2 – 4.9
50	2.2 – 2.6	4.0 – 4.6
60	2.1 – 2.5	3.8 – 4.4
70	2.0 – 2.4	3.6 – 4.3

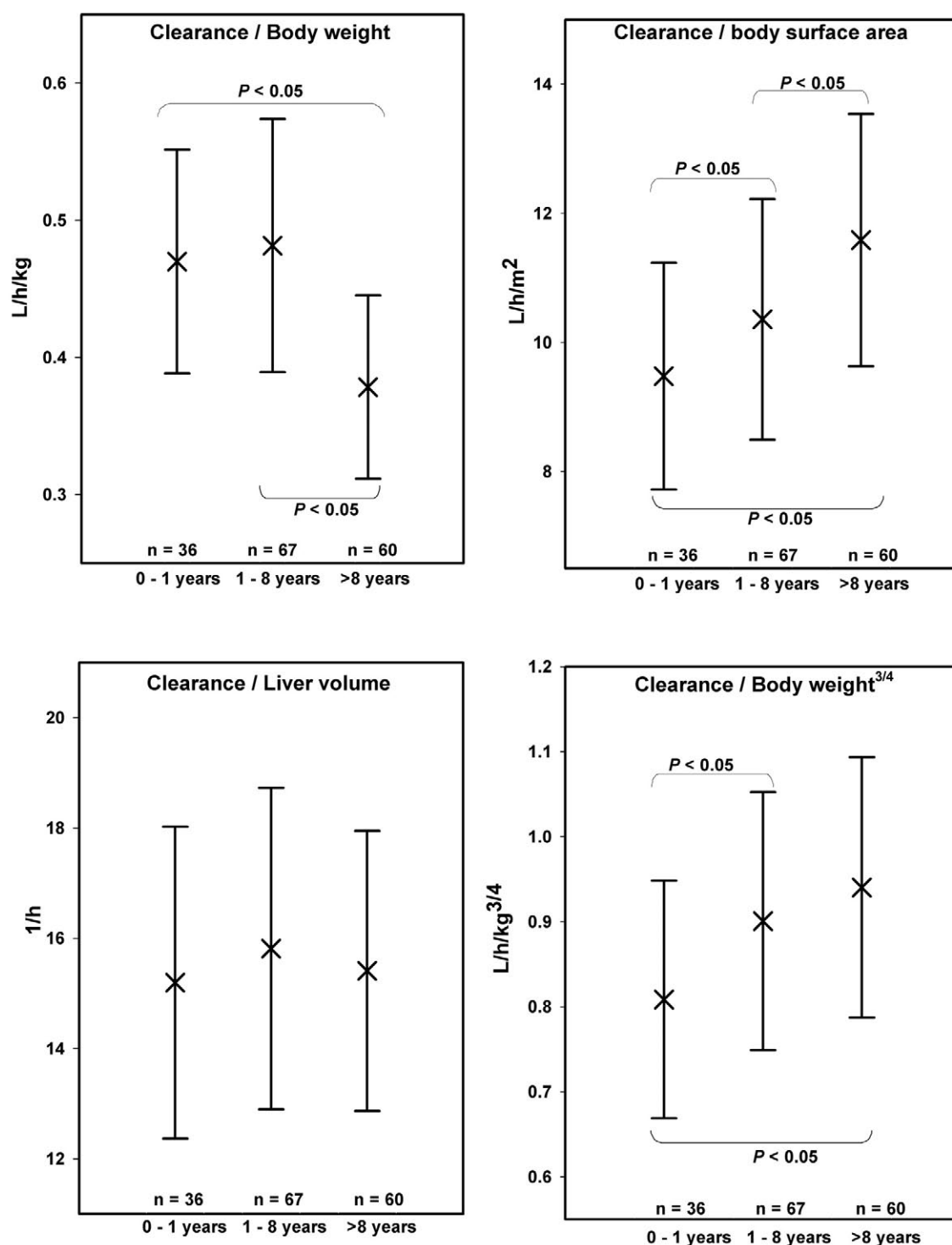
\*The dose requirement was calculated using the model based clearance (CL) and oral bioavailability (F) in the equation:  $\text{Dose} = (\text{CL}/\text{F}) \times \text{AUC}_{\text{req}}$ , where  $\text{AUC}_{\text{req}}$  is the mean daily area under the concentration-time curve (AUC) of cyclosporine corresponding to the typical target trough concentrations in the study population: the  $\text{AUC}_{\text{req}}$  of 19700 µg×h/L corresponded to a target trough of 300 µg/L and the  $\text{AUC}_{\text{req}}$  of 23000 µg×h/L corresponded to a target trough of 350 µg/L. For intravenous dosing the value of F was fixed to one. For the oral dosing it was assumed that the children are receiving the liquid microemulsion formulation of cyclosporine.

## 2. EMPIRICAL BAYES ESTIMATE BASED RESULTS

### Estimates of cyclosporine clearance, volume of distribution, and oral bioavailability (study I)

The empirical Bayes estimates of clearance, volume of distribution, and oral bioavailability were calculated and the estimates between different age groups were compared. The clearance/body weight (CL/BW) decreased with increasing age after the first year of life (Figure 9). The CL/BW was  $0.48 \pm 0.09$  L/h/kg (0.30–0.73 L/h/kg) in pre-pubertal children ( $\leq 8$  years), and  $0.38 \pm 0.07$  L/h/kg (0.19–0.64 L/h/kg) in older children. Very young patients (0.36 to 1 year,  $n=36$ ) had a similar CL/BW [ $0.47 \pm 0.08$  L/h/kg (0.33–0.67 L/h/kg)] to that of other pre-pubertal children (Figure 9). The estimates of liver volume (obtained by the empirically derived formula based on a meta-analysis: Liver volume =  $0.722 \times \text{BSA}^{1.176}$ ) (Johnson et al., 2005) were used to normalize clearance by liver volume. This scaling removed the relationship between age and clearance (Figure 9). The allometric weight model used to normalize clearance removed the relationship between age and clearance, but did so only for children after the first year of life (Figure 9). For patients older than one year, the allometrically scaled body weight explained about 96% of the variability in liver volume as assessed by simple linear regression. For patients younger than one year, the allometrically scaled body weight explained about 70% of the variability in liver volume. The body weight adjusted volume of distribution was  $2.35 \pm 0.65$  L/kg (1.26–4.61 L/kg) and did not change with age. The oral bioavailability was  $36\% \pm 8\%$  (with a range of 10% to 60%) and, likewise the volume of distribution, did not change with age.

Figure 9. The empirical Bayes estimates of clearance adjusted for body weight, body surface area (BSA), liver volume (LV), and allometrically scaled clearance (body weight<sup>3/4</sup>) in children awaiting renal transplantation (study I). The estimates for clearance are presented for three age groups: 0 – 1 years, 1 – 8 years, and > 8 years (mean  $\pm$  standard deviation).



The Xs indicate mean values, and the error bars indicate  $\pm$  standard deviation. The comparison between groups was carried out by using the *t*-test.

## RESULTS

### The pre-hepatic and hepatic extraction of cyclosporine (study II)

In study II the pre-hepatic and hepatic extraction of cyclosporine were calculated in renal transplant candidates. The pre-hepatic extraction ratio was  $0.51 \pm 0.13$  (n=58) with 3.7-fold variability between subjects (0.20 – 0.74), and the hepatic extraction ratio was  $0.24 \pm 0.04$  (n=98) with 2.4-fold variability between subjects (0.14 – 0.34). Linear regression analyses ascertained that the variability in the pre-hepatic extraction ratio explained 95% of the variability of cyclosporine oral bioavailability. These findings are presented along with previous publications in Table 9 for comparison.

**Table 9. Measures of cyclosporine\* oral bioavailability, hepatic extraction ratio, and pre-hepatic extraction ratio (Mean  $\pm$  SD) reported in published studies and in study II for oil-based and microemulsion formulations.**

n	Studied subjects	Adults/Children	Oral Bioavailability	Hepatic extraction ratio	Pre-hepatic extraction ratio	Reference
6	Healthy volunteers	Adults	$0.27 \pm 0.09$	$0.24 \pm 0.04$	$0.64 \pm 0.14$	(Hebert et al., 1992)
5	Healthy volunteers	Adults	$0.22 \pm 0.05$	$0.25 \pm 0.06$	$0.71 \pm 0.09$	(Gomez et al., 1995)
4	Renal transplant patients <sup>†</sup>	Adults	$0.36 \pm 0.09$	$0.27 \pm 0.1$	$0.51 \pm 0.2$	(Gupta et al., 1989a)
58	Renal transplant candidates	Children	$0.37 \pm 0.09$	$0.24 \pm 0.04$	$0.51 \pm 0.13$	Study II

\*In the previous publications the oil-based cyclosporine formulation was used. In study II the microemulsion formulation of cyclosporine was used.

<sup>†</sup>The study was conducted in stable renal transplant recipients 6–12 months after transplantation.

### The pre- to post-transplantation pharmacokinetic predictions (study III)

On the 3<sup>rd</sup> post-operative day, the post-transplantation systemic clearance correlated well with the pre-transplantation clearance ( $r^2=0.69$ ,  $P<0.0001$ , n=61) with a mean  $\pm$  SD prediction error of  $5.6\% \pm 10.8\%$ . Shortly after transplantation (5<sup>th</sup> postoperative day) the bioavailability did not correlate with the pre-transplantation bioavailability ( $r^2<0.01$ ,  $P=0.61$ , n=55), but later than one year after transplantation, the correlation was significant ( $r^2=0.20$ ,  $P=0.01$ , n=35). The prediction error of the 5<sup>th</sup> postoperative day bioavailability was highly variable,  $-2.0\% \pm 29.0\%$  (n=55). The oral clearance shortly after transplantation (5<sup>th</sup> postoperative day) correlated less markedly, but significantly, with the pre-transplantation oral clearance ( $r^2=0.16$ ,  $P=0.02$ , n=35).

### 3. THE PHARMACOGENETIC RESULTS

#### Cyclosporine pharmacogenetics in children awaiting renal transplantation (study II)

The frequencies of most of the studied DNA sequence variations were large enough (>7%) to detect a 15/30% change in clearance for heterozygous/homozygous patients, respectively. This level of change had a statistical power of over 80% at an alpha level of 5% (n=98). However, no significant associations, displaying a gene-dose effect, were found in the whole population for clearance, oral bioavailability or volume of distribution. In a further sub-group analysis, we found that in children older than eight years (n=22), the oral bioavailability of cyclosporine was  $0.28 \pm 0.07$  (0.19–0.34) in patients with the *ABCB1* c.2677GG genotype,  $0.36 \pm 0.07$  (0.27–0.48) in those with the c.2677GT genotype, and  $0.44 \pm 0.04$  (0.40–0.49) in those with the c.2677TT genotype ( $P=0.012$ ,  $r^2=0.372$ ). A similar, but weaker association was observed for the *ABCB1* c.1236C>T variant ( $P=0.032$ ,  $r^2=0.304$ ), which is in strong linkage disequilibrium with c.2677G>T. For the related c.1199G-c.1236C-c.2677G-c.3435C haplotype the association was weaker still ( $P=0.052$ ,  $r^2=0.268$ ). These differences were attributable to differences in the pre-hepatic extraction of cyclosporine as the variability in the pre-hepatic extraction ratio explained most of the variability observed for oral bioavailability.

#### Cyclosporine pharmacogenetics in children after renal transplantation (study III)

None of the *ABCB1* SNPs or haplotypes in the post-transplantation population was found to be associated with cyclosporine clearance or oral bioavailability in the whole population or in patients older than eight years. The only individual SNP that was found to be associated with cyclosporine pharmacokinetics was the *SLCO1B1* c.521T>C variant. Among patients younger than eight years (n=58), this variant was associated with a 10% per allele decrease in cyclosporine bioavailability ( $P=0.02$ ). However, no gene-dose effect associated with this *SLCO1B1* variant could be observed.

In the haplotype analyses of patients carrying the *NR1I2* g.-25385C–g.-24381A–g.-205\_-200GAGAAG–g.7635G–g.8055C haplotype, the bioavailability of cyclosporine was 11.2% (CI: 3.3% – 19.8%) lower, per allele, than for non-carriers ( $P=0.04$ ). This effect was also significant in a subgroup of patients older than eight years ( $P=0.01$ ). In contrast those patients carrying the *NR1I2* g.-25385C–g.-24381A–g.-205\_-200GAGAAG–g.7635G–g.8055T haplotype, the bioavailability was

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19.4% (CI: 5.3% – 31.1%) higher, per allele, than for non-carriers ( $P=0.01$ ). The effects of these *NR1I2* haplotypes on the bioavailability were consistent with a gene-dose effect.



## DISCUSSION

Studies I, II and III characterize the population pharmacokinetics of cyclosporine in 176 pediatric renal transplant recipients. A first order absorption, three-compartment pharmacokinetic model with: body weight (scaled allometrically), hematocrit, plasma cholesterol and serum creatinine as factors affecting clearance and volume parameters, along with time after transplantation and dosing frequency as factors affecting oral bioavailability, was used to describe the pharmacokinetics of cyclosporine. Before transplantation the carriers of the *ABCB1* c.1236C>T or c.2677G>T variant allele had about 1.3–1.6 times higher oral bioavailability for cyclosporine than non-carriers among children older than eight years. After transplantation, no *ABCB1* SNPs were found to covary with cyclosporine pharmacokinetics. However for carriers of the *NR1I2* g.-25385C–g.-24381A–g.-205\_-200GAGAAG–g.7635G–g.8055C haplotype, the bioavailability of cyclosporine after transplantation was about one tenth lower, per allele, than for non-carriers. This effect was also significant in a subgroup of patients older than eight years. In contrast, for patients carrying the *NR1I2* g.-25385C–g.-24381A–g.-205\_-200GAGAAG–g.7635G–g.8055T haplotype, the bioavailability of cyclosporine was almost one fifth higher, per allele, than in non-carriers. In other cases the variability in the pharmacokinetics of cyclosporine remained largely unexplained by the investigated sequence variations and haplotypes in the *ABCB1*, *ABCC2*, *SLCO1B1*, *CYP3A4*, *CYP3A5*, and *NR1I2* genes. Acknowledging the most influential factors affecting cyclosporine pharmacokinetics: body weight, time after transplantation, and dosing frequency, can help in cyclosporine dosage optimization in children.

### 1. METHODOLOGICAL CONSIDERATIONS

#### Pharmacokinetic modeling

Population pharmacokinetic modeling offers advantages in studying data that is part sparse and part rich (many samples/patient) (Davidian and Giltinan, 1995; Ette and Williams, 2004a). Our study data included rich pharmacokinetic data before transplantation i.e. frequent sampling after intravenous and oral cyclosporine test doses. After transplantation the data were mostly sparse, consisting of cyclosporine trough concentrations, C<sub>2</sub> concentrations, and occasional dose interval AUCs. It would not have been feasible to analyze changes of such pharmacokinetic data and

## DISCUSSION

explore covariate effects using noncompartmental or individual pharmacokinetic modeling. However, with population pharmacokinetic modeling we were able to obtain reliable population parameter estimates both before and after transplantation. This was possible, because the individuals “share” data in population modeling, which enables parameter estimation even for those individuals considered to have provided too few observations to allow parameter estimation by standard methods (Aarons, 1991). Model evaluation was performed throughout the model building process using: standard goodness-of-fit plots augmented by a visual predictive check (Karlsson and Holford, 2008) for the final model in study III. Thus, any large model-misspecification is unlikely to remain in the final model. Thus the population model should represent the pharmacokinetics of cyclosporine in renal transplanted children well.

In order to estimate all model parameters reliably, concentration time points after both intravenous and oral administration should be taken throughout the dose interval. Prior to transplantation the data were rich, with frequent sampling up to 28 hours from the beginning of the intravenous test dose and up to 24 hours after the oral dose. Therefore, we were able to reliably evaluate the tri-exponential characteristics of cyclosporine disposition. In previous studies that have compartmentally characterized cyclosporine pharmacokinetics, mostly two (Dunn et al., 2001) and sometimes three compartment (Kahan, 1989b) disposition models, have been presented (Dunn et al., 2001; Novartis, 2005). However, some studies comprising only trough data have even used a one-compartmental model to describe cyclosporine pharmacokinetics (Wu et al., 2005). The uni- and bi-exponential disposition models were found suitable in studies in which cyclosporine was administered orally and blood sampling was restricted to less than 24 hours. However, when blood sampling is frequent and the sampling time extends to 24 hours or even longer, the probability of detecting tri-exponentiality is high, as in study I.

After transplantation, the bulk of the data were available from 137 children and consisted of 3189 trough concentrations and 1044 C<sub>2</sub> concentrations. However, the exact times for C<sub>2</sub> ranged from one to four hours after the dose. Moreover, 63 dose interval AUCs were available in the post-transplantation time period. The  $T_{\max}$  of the microemulsion formulation of cyclosporine occurs about 1–2 hours after the dose (Holt et al., 1994; Friman and Backman, 1996). We modeled the absorption phase with a first-order equation without a lag-time. Such an approach has been used in previous published studies (Hesselink et al., 2004; Wu et al., 2005). However, some published

studies have described cyclosporine absorption by either a zero order process (Bourgoin et al., 2005) or by using the transit-compartmental absorption model approach (Debord et al., 2001; Rousseau et al., 2004). In the model building process, zero-order absorption and transit-compartmental absorption (Savic et al., 2007) were also assessed. However, these approaches were not found to be superior to the first-order absorption model. Moreover, a lag-time for absorption was estimated, yet in our population a lag in absorption was evident in only a few patients. Therefore a population parameter estimate for lag-time could not be obtained in our study. Furthermore, due to the lack of data on the absorption time period (there was no sampling between 0 and 1 hour post-dose), the early absorption phase could not be modeled with the same accuracy as the disposition model.

A great advantage in using the population pharmacokinetic approach is the opportunity it provides to model the inter-individual, inter-occasion, and residual variability separately. In study I, inter-individual variability could be estimated for all fixed effects parameters. However, in study III it was not possible to estimate the inter-individual variability for all parameters reliably due to the relative sparseness of our data in the post-transplantation time period. The coefficient of variation for the intravenous proportional residual error was 10% and for the oral proportional residual error it was about 30%. The additive residual error for intravenous cyclosporine dosing was 2 µg/L. The inherent variability in the assays used to quantify cyclosporine concentrations (within-run and between run coefficients of variation) was less than 10% for both FPIA and RIA methods. Thus, a significant proportion of the residual error, at least for oral cyclosporine concentration data, is likely to result from other factors, such as inaccuracies in data for dosing times and possibly missed doses.

In our studies individual parameter estimates were obtained using a population approach and Bayesian methodology, which yielded empirical Bayes estimates (EBEs) of the model parameters. The EBEs are estimates, which are informed by both prior population parameters and individuals' observations. Therefore the EBEs will approach the population parameter values, if the individuals do not provide sufficient observations to estimate the pharmacokinetic parameters separately. This dependency on the population parameters is referred to as  $\eta$ -shrinkage (Karlsson and Savic, 2007). In study I and II the  $\eta$ -shrinkage was low for: clearance, volume and oral bioavailability parameters, only 6% on average. Consequently, these values were not shrunk toward the

## DISCUSSION

population mean and did adequately reflect each individual's pharmacokinetic parameters. On the other hand, the EBEs calculated after transplantation (study III) had more  $\eta$ -shrinkage. Therefore, the EBE values obtained in study III (post-transplantation data) were more inclined to approach the population parameter values than those obtained in study I (pre-transplantation data). Unfortunately, due to methodological restrictions, the exact shrinkage values could not be obtained.

### **Cyclosporine assays**

An unfortunate limitation in our studies was the fact that two different analytical methods (specific radioimmunoassay and fluorescence polarization immunoassay) were used to quantify cyclosporine concentrations. In addition, two different and specific RIA methods were used. The problem with using RIA and FPIA together is that FPIA detects more metabolites than does the specific RIA, which results in 0–20% greater cyclosporine concentration values than those obtained by RIA (Morris et al., 1992; Aspeslet et al., 1997; Rodriguez et al., 2005). The assay method was modeled only as a residual error parameter, because the degree that the values differ from those measured by the RIA method is dependent on the individual's metabolite profile and the actual concentrations of these metabolites. The use of two slightly different RIA kits is also a point of concern. The residual error with the older RIA kit was estimated to be approximately 2.5 times that of the newer kit. However, by adding this factor as a covariate to the residual error, it could be adequately controlled.

A second limitation related to the analytical methodology was the observation that the clearance of cyclosporine would be about 20% less after oral administration than after intravenous administration. This finding was obviously an artifact. Oral administration of cyclosporine can result in somewhat higher concentrations than does intravenous administration (Kivistö, 1992; Safarcík et al., 2001) because more metabolites are formed after oral administration of cyclosporine (Aweeka et al., 1994), and also because the (specific) RIA method can cross-react slightly with cyclosporine metabolites. Although this was a problem that could not be overcome entirely by modeling, it could be minimized by allowing clearance to differ after either intravenous or oral administration.

The third limitation related to the analytical methodology was the fact that all immunoassays cross-react with cyclosporine metabolites to varying degrees. Therefore, the parameter estimates obtained by using one immunoassay are not directly comparable with the results obtained by using another immunoassay or HPLC (Johnston and Holt, 1999). For instance, there is no simple conversion factor that could be used to translate cyclosporine parameter estimates obtained by RIA to those obtained by HPLC. The greater the time interval between the dose administration and sampling, the higher the proportion of metabolites, and the higher the ratio of RIA to HPLC will be. Cyclosporine concentrations measured by HPLC are lower than those measured by RIA, and the cyclosporine clearance values presented in HPLC studies were higher than those in RIA studies (Dunn et al., 2001). However, RIA has been considered suitable for use as a reference method, and it seems to cause little bias compared to the HPLC assay (Johnston and Holt, 1999; Andrews and Cramb, 2002).

### **Pharmacogenetics**

A problem with many pharmacokinetic studies assessing genotype-pharmacokinetic phenotype associations, has been the difficulty to replicate the findings. Discordant results can result from a true lack of effect, or due to differences in study methodology (Leschziner et al., 2007). Important methodological issues include: the reliability of the phenotypic measure, of size and how representative the studied population(s) are, and also the mechanisms for controlling for the effects of multiple simultaneous covariates and confounding. When studying a pediatric population, pharmacokinetic differences due to age-related factors cannot always be distinguished from differences due to factors related to body size, because the effect of body size may mask the effects of other covariates (especially in a population with both young/small and older/larger children). Therefore, to identify covariates other than body size, such as genotypic or demographic characteristics, it is desirable to standardize or adjust these parameters to an appropriate body size measure, such as by allometric relationships (Meibohm et al., 2005).

In study I we found that allometrically scaled clearance could be used to explain developmental changes in the systemic clearance of cyclosporine. Moreover, we did not find any age-related changes in oral bioavailability. Therefore we were able to minimize the confounding effect of size and development on the estimates of clearance. Furthermore, the pre-transplantation pharmacokinetic data were obtained under carefully controlled and documented conditions,

## DISCUSSION

which minimized imprecision in the determination of pharmacokinetic parameters used for estimates (phenotype). However, imprecision due to inaccurate information about dosing and sampling times is unavoidable in studies based on post-transplantational routine therapeutic drug monitoring data. On the other hand, by using population pharmacokinetic analysis in the post-transplantation study, the effect of confounding covariate factors could effectively be minimized.

Our sub-group analyses of the different age groups illustrates a further potential challenge when interpreting the results of pediatric pharmacogenetic studies namely that: genotype-phenotype associations may only be apparent at certain ages or developmental stages. Accordingly, in the pre-transplantation time period, we found that carriers of the *ABCB1* c.1236C>T or c.2677G>T variant allele had about 1.3–1.6 times higher oral bioavailability and lower pre-hepatic extraction ratio of cyclosporine than non-carriers among children older than eight years. However, such an association was not seen in the younger age groups. When studying patient populations in the first years of life, it should be remembered that genotype-phenotype associations may only be apparent when the gene of interest is fully expressed.

## 2. CLINICAL AND GENETIC FACTORS AFFECTING CYCLOSPORINE PHARMACOKINETICS

### Factors affecting cyclosporine clearance and volume of distribution

Allometrically scaled body weight (body weight<sup>3/4</sup>) alone was responsible for 4-fold differences in uncorrected cyclosporine clearance and 6-fold differences in volume parameters. Clearance and volume parameters increased with increasing serum creatinine and decreasing plasma cholesterol and hematocrit, which combined explained up to 20% to 30% of the inter-individual variability of these parameters prior to transplantation. After transplantation these factors explained less of the inter-individual variability, as the variability in serum creatinine, plasma cholesterol and hematocrit also diminished.

Cyclosporine is a very lipophilic drug and is widely distributed throughout the body, the volume of distribution being 3–5 L/kg (Lill et al., 2000; Ptachcinski et al., 1986). As would be expected from its large volume of distribution, cyclosporine concentrations in body tissues are relatively high (Lensmeyer et al., 1991). Moreover, cyclosporine is extensively bound to blood and plasma constituents, with 90%–98% of the circulating cyclosporine bound to red blood cells and plasma protein (Akhlaghi and Trull, 2002). Cyclosporine is considered to have a low to medium hepatic extraction ratio and therefore its hepatic clearance is influenced by its unbound fraction in blood according to the well-stirred model (Pang and Rowland, 1977). As the clearance and volume of distribution of cyclosporine can be expected to increase with an increasing unbound drug fraction, it is logical to assume that plasma cholesterol and hematocrit values were significant covariates for both the clearance and volume of distribution of cyclosporine. In previous published studies, cyclosporine clearance was significantly less in patients with hyperlipidemia than in those with normal cholesterol levels in solid organ transplant patients (Awni et al., 1990). In another study, the volume of distribution at steady-state was negatively correlated with the concentration of total cholesterol (Gardier et al., 1993). In accordance with the findings of the present studies, hematocrit has been shown to covary with cyclosporine clearance in adult renal transplant recipients (Yee et al., 1988; Wu et al., 2005). Moreover, the typical values for clearance and volumes of distribution increased with rising serum creatinine levels in our population pharmacokinetic model. A possible explanation for this is that serum creatinine acts as a marker for factors that reduce cyclosporine blood binding (Lindholm, 1991a; Cooney et al., 1997).

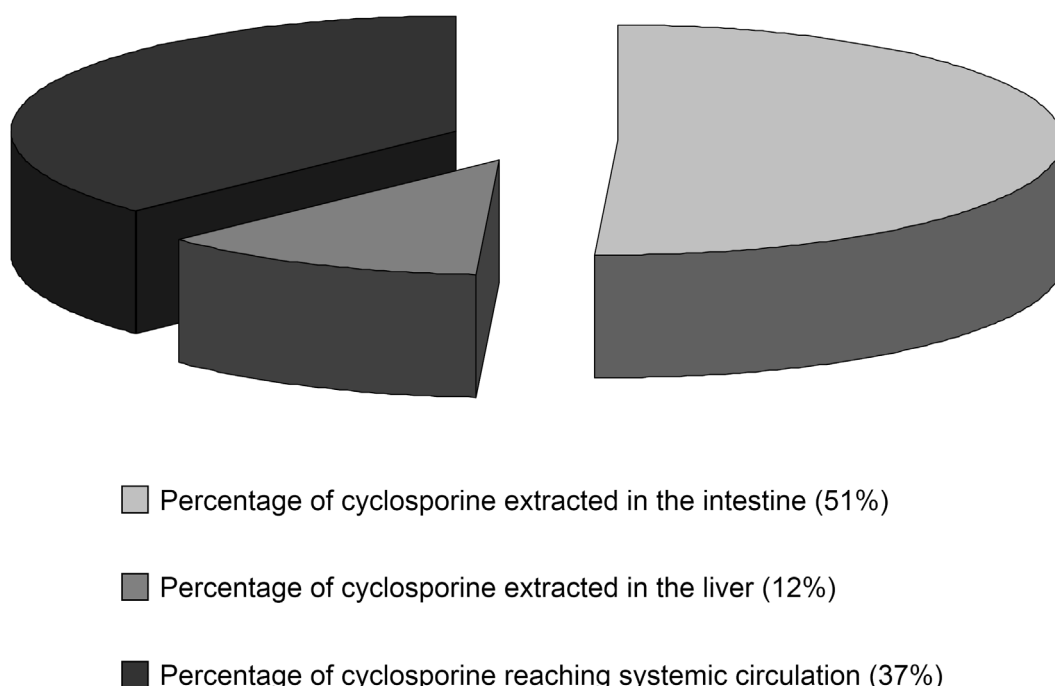
## DISCUSSION

### **Effects of pre-hepatic and hepatic extraction on cyclosporine oral bioavailability**

Cyclosporine undergoes extensive metabolism by CYP3A4 and CYP3A5 in the enterocytes of the small intestine and in the liver hepatocytes (Kelly and Kahan, 2002). In addition, CYP3A proteins are substantially expressed in the intestine, especially in the duodenum and the proximal jejunum (Kolars et al., 1994). Although the total CYP3A content in the intestine is less than in the liver, the tips of the duodenal and proximal jejunal villi are lined with mature CYP3A4-containing enterocytes which are readily exposed to drug molecules dissolved in the gastric and intestinal contents. This localization of CYP3A4 together with the findings that CYP3A4 content could be higher in intestinal enterocytes than in hepatocytes (von Richter et al., 2004) support the concept that drug metabolism in the intestinal wall substantially contributes to the overall first-pass metabolism of many CYP3A4 substrates, including cyclosporine (Kolars et al., 1991; Kivistö et al., 2004). As observed in the previous studies that used small numbers of patients (Table 9), the estimated pre-hepatic extraction ratio of cyclosporine in this study was high at over 0.5. Therefore, it was highly correlated with cyclosporine oral bioavailability ( $r^2=0.95$ ). Accordingly, the extensive cyclosporine metabolism by CYP3A4 and CYP3A5 in the enterocytes of the small intestine explains, the low oral bioavailability of cyclosporine to a large extent (Dunn et al., 2001; Kelly and Kahan, 2002) (Figure 10).



**Figure 10. Schematic depiction of the pre-hepatic and hepatic extraction of cyclosporine.**



#### **Developmental factors affecting cyclosporine pharmacokinetics**

Only few studies that assessed oral bioavailability of the microemulsion formulation of cyclosporine in the pediatric population have been conducted (Cooney et al., 1997; del Mar Fernandez De Gatta et al., 2002). Moreover, published data on young children ( $\leq 5$  years) are limited and virtually no data exist for patients younger than one year. In study I, we found that the range of cyclosporine clearance values adjusted for body weight gave higher values (0.2–0.7 L/h/kg) than those previously reported for adults (0.3–0.5 L/h/kg). The volume of distribution was similar or slightly smaller (1.3–4.6 L/kg) than in adults (3–5 L/kg). However, the oral bioavailability (10–60%) was within the range of adult values (Ptachcinski et al., 1985a; Ptachcinski et al., 1986; Hesselink et al., 2004; Wu et al., 2005). Oral bioavailability and volume of distribution adjusted for body weight did not change with age. However, pre-pubertal children ( $\leq 8$  years) had approximately a 25% larger clearance/body weight than did either older children (Figure 9) or adults (Ptachcinski et al., 1985a; Ptachcinski et al., 1986; Hesselink et al., 2004; Wu et al., 2005). These findings suggest that younger (0–8 years) children need about a quarter larger daily cyclosporine dose than do older children ( $\geq 8$ –18 years), to reach a similar cyclosporine concentration target. Moreover, because the volume of distribution/body weight did not change

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with age, it can be estimated that the half-life of cyclosporine is about 20% shorter in younger patients than in older patients.

The most plausible reason for the high hepatic clearance in young children is their large liver volume to body weight (Murry et al., 1995; Takahashi et al., 2000; Johnson, 2003; Strolin Benedetti and Baltes, 2003). After the liver enzymes and transporters have matured (CYP3A4 in the range of one year), the rate of liver metabolism is largely dependent on liver growth. Liver volume, blood flow and biliary excretion correlate well with the body surface area as an estimate of body size. However, very young children are not morphologically similar to adults. In comparison, infants have short legs, relatively big heads and large body trunks. Therefore, the body surface area equations are somewhat inaccurate in the youngest children (Mitchell et al., 1971). Another approach to estimate body size is to use the allometric method. Allometrics relate body function and morphology to body size (Holford, 1996). According to allometric principles for estimating the metabolic rate of the body i.e. clearance, the following equation is used:  $CL = CL_{\text{standard}} \times (\text{individual weight/standard weight})^{3/4}$ . The physiological basis of this approach is that the  $3/4$  power law for metabolic rates was derived from a general model that describes how essential substances are transported through space-filled fractal networks of branching tubes (West et al., 1999). Although the theory behind allometric scaling is complex, such models have been successfully applied to the pharmacokinetic modeling of many different substrates that undergo liver metabolism, such as paracetamol (Anderson et al., 2000), ciprofloxacin (Rajagopalan and Gastonguay, 2003), zidovudine (Capparelli et al., 2003), midazolam (Björkman, 2006), and alfentanil (Björkman, 2006). However, allometric scaling could not account for age-related changes in clearance in the youngest children for the aforementioned drugs and for cyclosporine in study I. The greatest disadvantage of the allometric scaling method is that it predicts clearance poorly in neonates and in infants in the first two years of life. In neonates and children under the age of one to two years, the maturing enzyme systems play a large role in the total variability not attributable to patient size (Johnson, 2005). Development, in a pharmacokinetic sense, can be viewed as comprising size-related and age-related changes. Although size-related changes are taken into account in the allometric model, age-related developmental changes that cause maturation in the enzyme systems remain unexplained.

In study I, allometrically scaled body weight removed age-related changes in clearance in patients older than one year. The reason for this finding could be, that in the youngest patients CYP3A4 maturation is incomplete, and therefore normalization for size-related changes cannot account for all developmental changes in the youngest patients. A further interesting finding was that when using the meta-analysis based liver size formula (Johnson et al., 2005) to normalize clearance, no age-related changes were detected, even in the youngest of patients (Figure 9). The liver-size scaling method was not used in study I, as the allometric scaling model is the most commonly used scaling approach recommended for population pharmacokinetic studies. However, as no age-related changes were seen in liver-size scaled clearance, the following question can be asked: Is CYP3A metabolism mature within the age of three to six months? Because no age-related changes in liver-size scaled clearance were detected, this finding suggests that CYP3A mediated liver metabolism of cyclosporine is already mature in the youngest patients. Furthermore, it can be speculated why the allometric method, which is used for normalization of size-related changes showed an age-related change in cyclosporine clearance. Although no clear answer is evident, a possibility is that the fixed  $\frac{3}{4}$  power exponent might not be correct in the youngest of patients.

For the patients of study I who were older than one year, the allometrically scaled body weight explained about 96% of the variability in liver volume (linear regression). However, the allometrically scaled body weight explained only 70% of the variability in liver volume of patients younger than one year. According to critiques of the allometric scaling method, the fixed  $\frac{3}{4}$  exponent should not be used. Instead this exponent value should be replaced by an estimated exponent, for a given drug (Mahmood, 2006). An advantage of the meta-analysis based liver-size equation is that it is not restricted by a theoretically fixed exponent, but rather is an empirical model based on a large (n=5000) database of children from birth to adulthood. In future studies, an interesting option would be to use liver volume based scaling for clearance instead of allometric scaling for drugs metabolized in the liver.

### **Cyclosporine pharmacogenetics in children**

In the whole pre-transplantation population (study II), the variability in cyclosporine pharmacokinetics remained largely unexplained by the investigated sequence variations and

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haplotypes, which suggests that genotyping for these variations is of limited clinical value in children. However, in patients older than eight years, we found that carriers of the *ABCB1* c.1236C>T or c.2677G>T variant allele had about 1.3 – 1.6 times higher oral bioavailability for cyclosporine than did non-carriers. However, after transplantation the *ABCB1* variations were not associated with cyclosporine oral bioavailability. Post-transplantation changes of physiological factors such as: an increase in CYP3A activity and a decrease in MDR1 expression (Nolin et al., 2008), as kidney function becomes normalized, could explain the lack of associations between *ABCB1* variants and cyclosporine pharmacokinetics after transplantation. However, another explanation could be that the original finding was a chance finding as it was based on a sub-group analysis of only 22 patients. Another alternative is that the finding is true and the therapeutic drug monitoring (TDM) data after transplantation contained unidentified confounding factors that render the original finding unreproducible.

MDR1 is situated in the luminal membrane of enterocytes and in the canalicular membrane of hepatocytes (Fromm, 2004). Due to the localization of MDR1 and CYP3A4 in the intestine, the function of MDR1 may allow CYP3A4 to have repeated and prolonged access to its substrate molecules and so limits the oral bioavailability of cyclosporine (Kivistö et al., 2004). Several previous studies in adults (Table 2) have investigated whether SNPs in the *ABCB1* gene are associated with cyclosporine pharmacokinetics. However, the results are discordant (Schwab et al., 2003; Hesselink et al., 2005a; Thervet et al., 2005) and there is no consensus on the clinical relevance of any of the studied *ABCB1* polymorphisms. The same conclusion can be drawn from our results.

In the post-transplantation analyses, no SNPs were found to covary with cyclosporine clearance or with oral bioavailability. Nevertheless, haplotype analysis revealed that the *NR1I2* g.-25385C–g.-24381A–g.-205\_-200GAGAAG–g.7635G–g.8055C haplotype caused a gene-dose dependent 11% (per allele) relative reduction in oral bioavailability of cyclosporine for the whole population (17% per allele reduction in patients older than eight years). In contrast, the *NR1I2* g.-25385C–g.-24381A–g.-205\_-200GAGAAG–g.7635G–g.8055T haplotype produced a 19% increase per allele in oral bioavailability of cyclosporine. The *NR1I2* gene encoding the pregnane X receptor may affect the expression levels of MDR1 and CYP3A4 (Zhang et al., 2008). The g.-25385C>T, g.-24381A>C,

g.7635A>G, and g.8055C>T variants have been associated with susceptibility to inflammatory bowel disease, which suggests that these variants are functionally significant or in linkage disequilibrium with a functional variant (Dring et al., 2006). Although the effect sizes attributable to these haplotypes are large, their usefulness in the clinical setting may be limited by stringent application of TDM.

In tacrolimus dosing studies, patients that expressed CYP3A5 needed about a 50% larger tacrolimus dose than did non-expressors (Hesselink et al., 2005a). It was hypothesized that CYP3A5 expressors were in risk of acute rejection after transplantation because of the possible delay of reaching therapeutic concentrations. In two separate studies, CYP3A5 expressors were found to have lower tacrolimus concentrations in the first week after transplantation than did CYP3A5 non-expressors (MacPhee et al., 2004; Hesselink et al., 2008). Despite this the delay in reaching therapeutic concentrations did not increase the incidence of acute rejections or tacrolimus toxicity (MacPhee et al., 2004; Hesselink et al., 2008). Nevertheless, the effect sizes attributable to the relatively common *NR1I2* haplotypes were large enough to warrant further studies.

### **Factors affecting cyclosporine pharmacokinetics after transplantation**

Pharmacokinetic studies suggest that the correlation between pre- and post- transplantation cyclosporine AUCs is generally poor (Lindholm and Kahan, 1993; Cooney et al., 1994b; Talaulikar et al., 2003). The authors of the above studies have hypothesized that changes after transplantation occur because of better absorption from the intestine (Freeman et al., 1985; Wilms et al., 1988; Kang, 1993). Another possibility is a decrease in the liver activity of the MDR1 because of subsiding uremia after transplantation (Nolin et al., 2008). In some relatively short studies in adult patients, the oral clearance of cyclosporine decreased by 30% in the first months after transplantation (Tufveson et al., 1986; Grevel et al., 1993; Felipe et al., 2003; Jacobson et al., 2003; Wu et al., 2005). In our study, the oral bioavailability of cyclosporine increased by about 1.3–1.5-fold in the first month after transplantation and thereafter gradually returned to the initial value of 39% and 29% after about 1.5 years, for b.i.d. and t.i.d. dosing, respectively. Increased absorption due to subsiding uremia cannot fully explain the time-course of the changes. For example, it can't explain

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why bioavailability returned to the pre-transplantation values slowly after transplantation. An interesting explanation is dose-dependent absorption or saturable pre-systemic extraction of cyclosporine. According to one study, systemic cyclosporine clearance is not saturated in the therapeutic concentration range (Gupta et al., 1989b). A study on adults administered the microemulsion formulation of cyclosporine gave results consistent with dose-linear pharmacokinetics, but only single doses that exceeded 3 mg/kg were studied (Müller et al., 1994b).

An increase of 30% in cyclosporine  $AUC_{0-24h}$  was reported when patients were changed from twice a day dosing (1.1 mg/kg single doses) to once a day dosing (2.2 mg/kg single dose) in 60 adult liver transplant patients (Kovarik et al., 2008). In a previous cross-over study on seven pediatric patients who received a daily dose of 5.7 mg/kg, as t.i.d. dosing produced nearly 40% lower daily AUC than did b.i.d. dosing (Filler et al., 2006). These findings suggest that cyclosporine pharmacokinetics is not dose-linear in the whole applied therapeutic dose range.

In study III, the patients on t.i.d. dosing constantly had about one fourth lower oral bioavailability for cyclosporine than did those on b.i.d. dosing. However, the daily doses were not similar in these groups, and patients on t.i.d. dosing received about a one third larger daily dose of cyclosporine. In our study, children on t.i.d. dosing were young and usually administered the liquid microemulsion formulation of cyclosporine, whereas children on b.i.d. dosing were older and received mainly the soft gelatine capsule microemulsion formulation. Unfortunately, it was not possible to extract reliable formulation data from ambulatory patients. Thus, it cannot be ruled out that the observed bioavailability difference between b.i.d. and t.i.d. dosing is explained by factors related to the use of the two cyclosporine formulations or to patient age.

In the first month after transplantation the average daily cyclosporine dose ranged from 11 to 15 mg/kg. The largest doses of cyclosporine were administered in the first 3–6 months after transplantation. Thereafter the single doses of cyclosporine were often less than 3 mg/kg. It can be hypothesized that the inhibitory effects of cyclosporine on CYP3A4 and MDR1 (Goldberg et al.,

1988; Combalbert et al., 1989; Fricker et al., 1996; Kajosaari et al., 2005) result in auto-inhibition and dose-dependent pharmacokinetics at the low single doses used in our patients. In contrast, complete saturation of CYP3A4 and MDR1 at higher doses renders cyclosporine pharmacokinetics dose-linear after a certain threshold dose (Lee et al., 2005). In addition to the systematic change in oral bioavailability, a factor that explains the poor correlation of pre- and post-transplantation cyclosporine AUCs was the large inter-occasion variability in oral bioavailability (CV $\approx$ 20%). The causes for this variability are likely to include: fluctuations in physiological parameters, such as intestinal motility and mesenteric and hepatic blood flow, early after transplantation. Other explanations are: the variability in analytical methods, inaccuracies in data for dosing times, and possibly missed doses.

### 3. CLINICAL IMPLICATIONS

Therapeutic drug monitoring of cyclosporine strives to minimize cyclosporine exposure and to avoid cyclosporine toxicity while maintaining adequate cyclosporine concentrations to guard against rejection. To optimize cyclosporine dosing and therapeutic monitoring in children it is important to understand how developmental, genetic, and other clinical factors contribute to variability in cyclosporine pharmacokinetics.

Body weight, scaled allometrically, was the most significant covariate for clearance and for volume parameters. This covariate alone was responsible for 4-fold differences in cyclosporine clearance and 6-fold differences in volume parameters. In addition, clearance and volume parameters increased with increasing serum creatinine and with decreasing plasma cholesterol and hematocrit. These covariate effects were estimated to explain inter-individual differences in clearance and volume of distribution of up to 20–30% before transplantation. However, after transplantation the mean serum creatinine value decreased by 85% and the variability in serum creatinine, hematocrit and plasma cholesterol was much less than before transplantation. Therefore, the clinical significance of these covariates after transplantation was small.

Currently, a pharmacogenetic test performed prior to transplantation for the individualization of cyclosporine dosing does not seem useful, as the bioavailability and clearance of cyclosporine remained largely unexplained by most of the genetic variations studied. Consequently, more studies are required on the functional significance of *ABCB1* SNPs. Furthermore, after transplantation, the effects attributable to the relatively common *NR1/2* g.-25385C–g.-24381A–g.-205\_-200GAGAAG–g.7635G–g.8055C and the *NR1/2* g.-25385C–g.-24381A–g.-205\_-200GAGAAG–g.7635G–g.8055T haplotypes could be large enough to be clinically significant, which warrants further study.

Pre-pubertal children ( $\leq 8$  years) had approximately 25% greater clearance/body weight than did older children or adults. However, oral bioavailability was similar for all age groups. Taken together these findings imply that younger (0–8 years) children need about a quarter larger daily cyclosporine doses than do older children ( $\geq 8$ –18 years), to reach similar cyclosporine concentration targets. Moreover, because the volume of distribution/body weight did not change with age, it can be estimated that the half-life of cyclosporine is about 20% shorter in younger



patients than in older patients, which leads to greater peak-to-trough ratios. To avoid high cyclosporine peaks associated with acute nephrotoxicity (Perico et al., 1992) and tremor (David-Neto et al., 2000), the daily dose can be divided in three single doses in young children. However, in doing so, an important finding to remember is that, in our study, the older children who received the capsule microemulsion formulation of cyclosporine as b.i.d. dosing had about 1.25–1.3 times higher oral bioavailability for cyclosporine than did younger patients who received the liquid microemulsion formulation as t.i.d. dosing. Moreover, the oral bioavailability increased by about 1.3–1.5-fold in the first month after transplantation, and gradually returned to initial values after approximately 1.5 years. These changes necessitate individual dose modifications and in particular frequent monitoring during the first months after transplantation.

Because cyclosporine dosing is guided by therapeutic drug monitoring, it is important that the magnitude of the inter-occasion variability be estimated and taken into account in dose modifications. The inter-occasion variability of oral bioavailability was about the same extent as that of the inter-individual variability ( $CV \approx 20\%$ ). Such a high inter-occasion variability further reduces the value of infrequent therapeutic drug monitoring. This can be due to either a change in the monitored concentration level indicating a true change in the individual pharmacokinetics of a patient, or a false change due to random inter-occasion variability. Therefore, frequent cyclosporine concentration monitoring and the use of two or more monitoring occasions when making dose modifications should be sought.

The inter-occasion variability was also the major factor that caused the poor pre- to post-transplantation predictability of the oral bioavailability. The systemic clearance immediately after transplantation correlated strongly with the pre-transplantation clearance ( $r^2=0.69$ ), as opposed to the oral bioavailability, which correlated poorly with its pre-transplantation value ( $r^2<0.01$ ). These findings suggest that an oral pre-transplantation pharmacokinetic study, for the calculation of the starting doses of cyclosporine, is not feasible. On the other hand, an intravenous pharmacokinetic study could be used to rapidly achieve the target concentration with intravenous cyclosporine dosing. Thereafter, the switch to oral dosing can be achieved assuming that the oral bioavailability increases to 40-50% during the first week. Alternatively, the model-predicted doses (Table 8) can be used to estimate the intravenous and oral cyclosporine starting doses after transplantation.

## DISCUSSION

This study described comprehensive long-term pharmacokinetic analyses of cyclosporine in renal transplanted children. It describes in detail a first order absorption, three-compartment pharmacokinetic model with body weight, hematocrit, plasma cholesterol and serum creatinine as factors affecting clearance and volume parameters. Moreover, and time after transplantation and dosing frequency as factors affecting oral bioavailability, were used in the model to describe the pharmacokinetics of cyclosporine. This model can be used to anticipate the need for individual dose modifications *a priori*. However, due to variability between and within patients that remains unexplained by the studied covariate effects, *a posteriori* dose adaptation by therapeutic drug monitoring remains indispensable in cyclosporine therapy. In the future our aim will be to incorporate clinical end-point data (glomerular filtration rate measurements and acute rejection data) to the pharmacokinetic data and gain insight to the optimal therapeutic cyclosporine concentrations. Thereafter, the concentration measurements of a given patient could be used in the population model to produce individual empirical Bayesian parameter estimates. These individual parameters could then be used to forecast the outcome of a dose adjustment, and the likelihood of reaching a specified concentration target could be maximized.

## CONCLUSIONS

A 3-compartment population pharmacokinetic model that had first order absorption without lag-time was used to describe the time course of cyclosporine kinetics in children. The most important factor that affected systemic clearance and distribution volume was allometrically scaled body weight. Pre-pubertal children (age  $\leq 8$  years) had a 25% larger cyclosporine systemic clearance/body weight (L/h/kg) than did older children. However, adjusting clearance for allometric body weight (body weight<sup>3/4</sup>) removed its relationship to age after the first year of life. The relationship between clearance and allometrically scaled body weight is consistent with a gradual reduction in relative liver size, to that of adult values, and a relatively constant CYP3A content in the liver from about 6–12 months of age to adulthood.

In contrast to cyclosporine clearance related to absolute body weight, the oral bioavailability was similar in all age groups, which implies that younger children need about a quarter larger daily cyclosporine dose than do older children ( $\geq 8$  years). Because the volume of distribution/body weight did not change with age, it can be estimated that the half-life of cyclosporine is about 20% shorter in younger patients than in older patients, which leads to greater peak-to-trough ratios. Therefore, to avoid high cyclosporine peaks, thrice daily dosing may be necessary in younger children. However, after transplantation the oral bioavailability was 1.25–1.3 times higher in the older children receiving cyclosporine twice daily as the gelatine capsule microemulsion formulation, than in the younger children receiving the liquid microemulsion formulation thrice daily.

Cyclosporine clearance and volume of distribution did not change with time after renal transplantation. However, cyclosporine oral bioavailability increased over 1.5-fold in the first month after transplantation, returning gradually thereafter to its initial value in 1–1.5 years. The largest cyclosporine doses were administered in the first 3–6 months after transplantation and thereafter the single doses of cyclosporine were often less than 3 mg/kg. Thus, the results suggest that cyclosporine already displays dose-dependent, saturable pre-systemic metabolism at low single doses, whereas complete saturation of CYP3A4 and MDR1 (P-glycoprotein) renders cyclosporine pharmacokinetics dose-linear at higher doses.

## CONCLUSIONS

Cyclosporine pharmacokinetics remained largely unexplained by most of the genetic variations studied both before and after renal transplantation. However, before transplantation, in children older than eight years heterozygous and homozygous carriers of the *ABCB1* c.2677T or c.1236T alleles had about 1.3 times or 1.6 times higher oral bioavailability, respectively, than did non-carriers. After transplantation, none of the *ABCB1* SNPs or any other SNPs were found to be associated with cyclosporine clearance or oral bioavailability in the whole study population or in the patients older than eight years. Although the post-transplantation findings strongly suggest that *ABCB1* polymorphisms do not affect cyclosporine pharmacokinetics in children, the results obtained prior to transplantation suggest that, in children, genotype-phenotype associations may only be apparent when the gene of interest is fully expressed. Therefore, developmental changes should be taken into account in pediatric pharmacogenetics studies.

After transplantation, the bioavailability of cyclosporine in patients carrying the *NR1I2* g.-25385C–g.-24381A–g.-205\_-200GAGAAG–g.7635G–g.8055C haplotype was about 11% lower, per allele, than in non-carriers. This effect tended to be more prominent in a subgroup of patients older than eight years. Furthermore, the bioavailability in patients carrying the *NR1I2* g.-25385C–g.-24381A–g.-205\_-200GAGAAG–g.7635G–g.8055T haplotype was almost one fifth higher, per allele, than in non-carriers. Currently, genotyping for any of the studied DNA sequence variations in order to individualize cyclosporine dosing cannot be recommended, because cyclosporine pharmacokinetics is not in harmony with most of the studied genetic variations. However, the effect of *NR1I2* genotype on cyclosporine pharmacokinetics warrants further study.

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